Capillary Electrophoresis: Lecture Notes

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MFRC Midwest Forensics Resource Center

Capillary Electrophoresis

Preface

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About the Presenter

Dr. Edward Yeung earned his A.B. degree in chemistry from Cornell University in 1968 and his Ph.D. in chemistry from the University of California at Berkeley in 1972. Since then he has been on the chemistry faculty at Iowa State University, where he is currently Distinguished Professor in Liberal Arts and Sciences.

Dr. Yeung's research interests span both spectroscopy and chromatography. He has published in areas such as nonlinear spectroscopy, laser-based detectors for liquid chromatography, capillary electrophoresis, trace gas monitoring, single-cell and single-molecule analysis, DNA sequencing, and data treatment procedures in chemical measurements. Dr. Yeung is an associate editor of *Analytical Chemistry* and served on the editorial advisory board of *Progress in Analytical Spectroscopy, Journal of Capillary Electrophoresis, Mikrochimica Acta, Spectrochimica Acta Part A, Journal of Microcolumn Separations, Electrophoresis, Journal of High Resolution Chromatography, Chromatographia,* and *Journal of Biochemical and Biophysical Methods.*

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Dr. Yeung was awarded an Alfred P. Sloan Fellowship in 1974, was appointed Honorary Professor of Zhengzhou University and of Zhongshan University, PRC, in 1983 and in 1995, respectively, and was elected Fellow of the American Association for the Advancement of Science in 1992. He received the ACS Division of Analytical Chemistry Award in Chemical Instrumentation in 1987; R&D 100 Awards in 1989, 1991, 1997, and 2001; the Lester W. Strock Award in 1990; the Pittsburgh Analytical Chemistry Award in 1993; the L. S. Palmer Award in 1994; the ACS Fisher Award in Analytical Chemistry in 1994; the Frederick Conference on Capillary Electrophoresis Award in 1997; the Eastern Analytical Symposium Award in 1998; the ACS Award in Chromatography in 2002; and the International Prize of the Belgian Society of Pharmaceutical Sciences in 2002.

Capillary Electrophoresis

1. Basic Mechanism and Operation

This guide is intended to accompany the *Capillary Electrophoresis* DVD and assumes some basic knowledge of chemistry or biochemistry. If you are a practitioner who uses commercial instruments in a laboratory and analyzes results, understanding the basic principles that govern limitations and capabilities is an important part of your training. This resource guide is designed as a quick reference for those interested in capillary electrophoresis and follows the DVD step by step.

Learning Objectives

At the end of this section, the viewer should be able to:

- Describe electrophoresis.
- Explain conditions in which you would use capillary electrophoresis.
- State advantages and disadvantages of capillary electrophoresis.
- Explain electroosmotic flow and how it is a factor in capillary electrophoresis.
- Describe how micelles separate neutral compounds in micellar electrokinetic chromatography.
- State the advantages of using MEKC.

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Introduction

Capillary electrophoresis (CE) is a newer separation technique that may have its beginnings about 1979 but became more widespread in the mid-1980s. It has since received a substantial boost, both in funding and research, because of the Human Genome Project*, where capillaries were used.

Separations are done in quartzlike, fused-silica capillary tubes that are approximately 50 to 150 cm in length. A microscopic hole runs all the way through the capillary tube; the internal diameter of the hole can be from 25 to 100 μm. If you think of a pencil or pen mark as being 1 mm, 100 μm is one-tenth to one-fortieth of that in size. The outside diameter of the capillary tube is generally between 150 to 350 μm or 0.15 to 0.35 μm.

Quartz is clear; the outside of the capillary tube is slightly brownish in color because it has a polymer coating. The coating, a polyimide, is an organic material, like Teflon®. The coating is needed because quartz is extremely rigid and would easily break with handling. The polymer provides a protective coating, so the tube can be bent without breaking; tubes also can be made of glass. The tubes are relatively inexpensive, a few dollars per meter.

A small window is created by burning off some of the organic polymer. That area of the tube then becomes very fragile during operation because it is no longer protected. Light is transmitted through this window for viewing the absorption of light or fluorescence due to excitation.

To help in separation, the medium inside the tube is generally water with salt or a pH-type buffer. Water is the solvent and dissolves the material being analyzed; salt allows electrical conductivity so voltage can be applied.

The voltage applied can be up to 30 kV—very, very high voltage—but because the tube is so small, there is little electrical current. The total electrical power going through the tube is about 10 W. Compared to a small home space heater, which is several hundred watts, 10 W does not heat the tube even with such high voltage running across it.

*To learn more about the Human Genome Project, see http://www.ornl.gov/TechResources/Human_Genome/project/about.html

Working with kilovolts sounds dangerous, considering a wall plug is only 110 V, but it is the current, not the voltage, that is the hazard. The power supplies used in capillary electrophoresis could give an electrical shock, and possibly a secondary problem if one had a preexisting heart condition, but all manufacturers have developed safety interlocks and other features to terminate power in dangerous situations.

A 30 kV, 10 W power supply is used to drive the cathode ray tube that provides a picture like a typical television. In fact, in the early days, researchers took power supplies from old TVs and purchased a capillary tube for about $10 to do electrophoresis experiments. Capillary electrophoresis became popular because of its tremendous powers in separation and because it was relatively easy and inexpensive to use.

To do an experiment, a high voltage power supply is connected to the tube. Solution vials are needed because the solution becomes the contact point; as in electrolysis or electrochemical experiments, voltage can be applied across it.

To inject the sample, the capillary tube is dipped into the sample vial while the voltage is disconnected. At the same time, some of the sample can be pushed into the capillary tube either by drawing to a vacuum on the other side, by applying positive air pressure, or by using electrical current. Unlike gas chromatography (GC) or liquid chromatography (LC), where a syringe and pressure are used to inject a certain volume of material directly into the column, the injection is done separately—the column is not interrupted because the column is rigid.

All three modes used in the process, whether by hydrodynamic or electrokinetic injection, are generally implemented in a commercial instrument with a computer-controlled screen. The cost of such an instrument is somewhere between $30,000 and $60,000 (Figure 1).

In a home-built situation, such an experiment would be a basic $100 setup, using a Radio Shack® power supply and capillary tube.

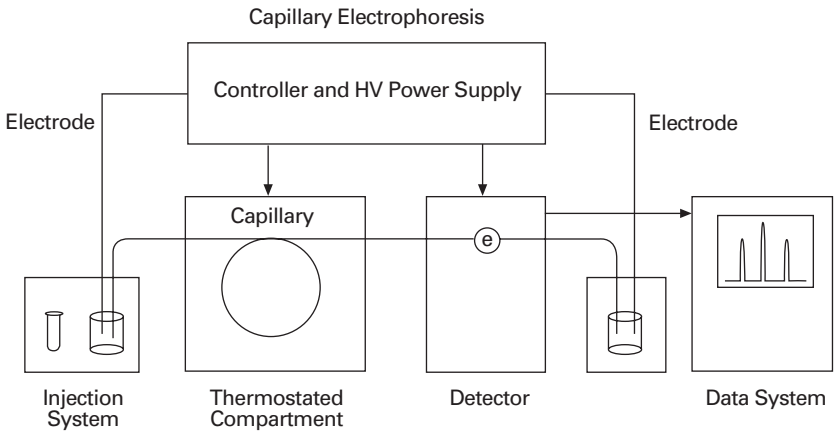

Figure 1. Schematic diagram of a capillary electrophoresis system.

Liquid chromatography uses absorption detectors and fluorescence detectors, and those, even though they are not designed for these very, very small capillaries, have been used since the early days of capillary electrophoresis. With a similar detector, the plot of absorption versus time is just like that in liquid chromatography.

In gas chromatography, it would be signal versus time so all these chromatograms look alike. In electrophoresis, just to emphasize the fact that it is electrophoresis, the plot (gram) is called an electropherogram.

Electrophoresis is a phenomenon where electric fields are used to move ions around. Biochemists have been using electrophoresis for many decades in separating proteins and even DNAs with slab gels. With slab gels, material is put in a large planar format and separations occur when an electric field is applied.

Traditional electrophoresis separations are done in 1 mm tubes instead of the 25 μm tubes used in capillary electrophoresis. To move the analyte over the same distance, capillary electrophoresis is roughly 20 times faster. The voltage is very high, and because it is high, things move faster because the driving force is larger.

The resolution, the ability to resolve peaks, is about four times better. Because of these two characteristics—speed and resolution—and the Human Genome Project with its large number of DNA sequences that could be read 20 times faster with higher resolution, capillary electrophoresis had its advantages.

Another major advantage is the sample size. In liquid chromatography or gas chromatography, an injection requires 1–5 µl of sample. *Microliters.* In capillary electrophoresis, the amount of sample is somewhere between 10 and 100 nl. *Nanoliters.* The sample is 100 times or 1,000 times smaller in volume—a very small amount of material. For use in forensic laboratories, this means the sample does not need to be diluted to get the 1–5 µl sample required. The very small volume necessary (e.g., that tiny drop of blood you may have), can be injected directly into the system.

Even though capillary electrophoresis uses 10–100 nl of sample, its full potential has not been reached. In order to pipette, some of the vials actually start up with 1 µl or more; only a small fraction of that is used. There are ways of pipetting things down to the 50 nl range; technology will eventually allow very small samples.

In high performance liquid chromatography (HPLC), the pump runs at about 1 ml/min, depending on the number of samples and how long the chromatogram is. It uses solvent, so waste solvent is collected; those solvents are generally not very environmentally friendly.

In capillary electrophoresis, the rate is 10–100 nl per second—perhaps half a microliter per minute. Half a microliter compared to 1 ml/min is a significant difference.

In the course of an experiment, or in an entire day, only a small bottle of waste material would be collected. The material would be mainly water because electrophoresis is done in water with some salt, like phosphates, which are essentially environmentally tolerable.

Those are the advantages, but there are some disadvantages when comparing capillary electrophoresis with traditional electrophoresis or gas or liquid chromatography. Capacity is a disadvantage; simply, a lot of material cannot be put into the system. Capillaries cannot handle an industrial-scale separation, purifying grams or kilograms of material.

For forensics work, however, analysis is the focus not capacity; small samples are desirable. Detection affects analysis. Because of such small dimensions and such small material, every kind

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of detector will fail gradually when going to smaller and smaller amounts of material and smaller and smaller dimensions. New detectors have been invented throughout the process of developing capillary electrophoresis to better detect the material after separation.

Liquid chromatography detectors were used initially to make it through transition; soon fancy optics were developed to look at capillaries very efficiently and with good sensitivity.

Optical clarity or cloudiness is generally not an issue. The capillary is only 50 μm in diameter, and the particles for clay- or salt-type samples are much larger than 50 μm and would not go inside. Something like milk, which is colloidal, cannot be put inside a capillary because of light scattering, but there are also other detectors. Fluorescence detectors can tolerate a certain amount of light scattering. Capillary electrophoresis is water based and, therefore, it is a transparent process.

In a free solution, meaning in a normal water-type solution, you can do electrophoresis, and that kind of electrophoresis is called zone electrophoresis or capillary zone electrophoresis (CZE). A zone of sample is injected, and this zone of sample is separated from other zones of different analytes. This is the simplest form of electrophoresis.

The separation occurs when force is applied to the analyte. The analyte is the known material to be separated from the other components. The force on any particle, which includes the ions or analytes in the solution, is equal to the charge of that particle multiplied by the electric field.

The electric field is in units of volts per centimeters (i.e., 30 kilovolts applied across 30 centimeters is 1,000 volts per centimeter—the electric field). The higher the electric field, the more force will be imparted on the analyte.

Zone (Free Solution) Electrophoresis (CZE)

+ force = qE
charge q coulombs, field strength E V/cm
– force = fv

Example: q is the charge of the particle, so with chloride ions, Cl^-, there is one charge; q will be the one coulomb of charge on one mole—or one electron's worth per ion. Sulfate, SO_3^{2-}, has two charges, so it will experience twice the force of a chloride ion.

Separating chloride from sulfate is a very simple separation because one is experiencing twice the force of the other; sulfate will move twice as fast as chloride.

A charge also cannot be equal to integers; such is the case with weak acids and bases. A simple weak acid, such as acidic acid, at very low pH is neutral, protonated, has no charge, and will experience no force.

At high pH—pH of 7.0, 8.0, 9.0—one of the protons will leave the acidic acid, so what is left is one negative charge on the acidic acid entity, and that is one charge.

But in between, near the pK of the acid dissociation constant of acidic acid, which is about 4.5 or so, there is a fractional charge—it does not go from 0 to 1, and can have a 0.5 charge. A 0.5 charge does not mean that an acidic acid can have half an electron; it means that on the average, or 50% of the time, the acidic acid will have an electron and 50% of the time it will not have an electron. On the average, it has a one-half unit charge, and that is why q can be 0.5 or 0.1, depending on where it is on the titration curve.

A useful control of capillary electrophoresis is by changing the pH; the fractional charge of any weak acid or weak base can be changed, and, therefore, it can move faster or slower.

With two weak acids, one with a pK of 4.0 and one with a pK of 5.0, choose a pH of 4.5—in the middle. The one with a pK of 4.0 will be effectively more negatively charged, and the one with a pK of 5.0 will be less negatively charged. One number will be larger than 0.5 and one number smaller than 0.5, so they can be separated very well.

An easy way to control the separation of typical organic compounds with any kind of functional group is simply change the pH if it does not separate. This is easier to understand and easier to

implement than in liquid chromatography where there are organic solvents that need to be mixed to the right polarity and gradient separations are done.

While there is the driving force moving ions from the injection site to the detector, there is some retardation force. The retardation force is because as anything moves, other than in a vacuum, it will suffer some collisions. For example, if you are moving in water, trying to swim, the water is retarding you. It is the frictional drag caused by water molecules.

The frictional drag depends on the size of the molecule and depends on its velocity. For example, if you drive down the freeway, the faster you go, the more wind resistance your car will experience; with a larger vehicle, you will experience more frictional drag than with a smaller or more streamlined vehicle.

This force acting against motion can be controlled by changing the viscosity of the solvent. Large molecules can be separated from small molecules very easily, simply because the large molecules tend to move slower.

frictional coefficient $f = 6\pi\eta R$ hydrodynamic radius

$m\,(dv/dt) = qE - fv = 0$ at steady state $v_{ss} = qE/f$

electrophoretic mobility $\mu = q/f$
f is function of molecular weight (MW)

The frictional coefficient has to do with the viscosity of the solution, and the hydrodynamic radius is how large the molecule is; the frictional force on it can be calculated.

At any one time, the mobility of the molecule—how fast the molecule will move—depends on the charge. It will move faster if there is more charge and slower if there is more drag.

It is the interplay, the tradeoff, between driving force q, the charge, and frictional force f, that is causing a particular species to move at a certain speed. Frictional force is a function of molecular weight; the more molecular weight, the larger the molecule. It is not difficult to optimize a capillary electrophoresis separation because this interplay is the only mechanism present in separating

molecules in free solution electrophoresis. It is simpler than in liquid chromatography, which has hydrophobic interaction, charge interaction, charge pairing signs, and so forth.

The one thing different in capillary electrophoresis from all the other kinds of chromatography is electroosmotic flow.

Electroosmotic Flow
Wall charged, thus counter-ion rich electrical double layer

See Figure 2.

electrical potential
K^{-1} is double-layer thickness (1/e point) dependent on solution
only valid for $\psi_o < kT/\varepsilon = 25.7$ mV
ε is permittivity
surface charge density $\sigma^* = \varepsilon K \psi_o$

Figure 2 is a plot of the charge of the electric potential at the surface of the capillary tube, so the left-hand side is the capillary tube. On the right side of the axis is the solution.

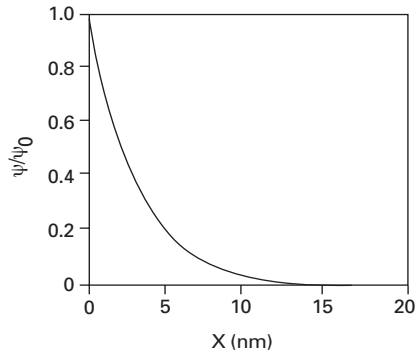

Figure 2. Plot of the electrical potential, ψ, as a fraction of the surface potential, ψ_o, as a function of the distance from the capillary wall, X. A value of $3.29 \cdot 10^8$ m was used for κ.

The plot shows that the electrical potential at the wall is very high, and the electrical potential in a solution away from the walls is essentially 0. This distance is in the order of nanometers not microns. The tube is 25–100 μm, so this is a very thin layer of electrical potential, right next to the wall of the fused silica. Electrical potential is found because fused silica, unlike Teflon®, is not totally inert, and it actually has replaceable hydrogens on the surface.

At normal conditions, some of those hydrogens will be dissociated, leaving a negatively charged surface—a negative charge due to the presence of dissociation on the capillary tube itself.

Because of the randomization of water molecules, the water molecules shield the rest of the molecules farther and farther away from the wall so that they do not feel the effect of the charge. Only the very surface layer will feel the charge.

While that sounds irrelevant, it creates a highly undesirable effect in capillary electrophoresis because if there is a charge and an electric field is applied, there will be some force driving the liquid right at the surface. Even though the water molecules are not charged, there is a surface layer of charge that is due to the silanol groups dissociating, and those charges will be influenced by the same electric field. This can be thought of as a sort of skin of liquid around the central tube of bulk liquid that contains your solution.

The skin is being driven by the electric field, and if the skin is driven by the electric field, it will actually carry the rest of the water molecules with it.

This skin or sheath that is moving down carries the water molecules. If something neutral is injected (e.g., methanol), methanol will move along the capillary tube, not because methanol is experiencing any electric field charge driving force but because the skin of this tube of water inside is being pushed. If the skin is pushed, the rest of the water and the injected methanol also move; even neutral molecules move.

The wall is charged, and therefore, the ions around this charged region interact with the electric field so that everything moves with electroosmotic flow.

Electroosmotic Velocity

$$v(x) = \frac{-E\,\sigma^*}{K\eta}\,[1 - \exp(-Kx)]$$

fused silica $\sigma^* \sim 0.01$ C/m^2, $K = 3.29 \times 10^8$ m^{-1}

E = 30 kV, $\eta = 0.001$ N s/m^2, 99% at 14 nm plug flow

See Figure 3.

$\mu_{eo} = -\varepsilon\psi_o/\eta = \sigma^*/K\eta \sim 6.1 \times 10^{-8}$ m^2/Vs

volume flow rate (50 μm) 3.5 nl/s
comparable to 3.4 psi/m pressure flow

Figure 3 is a plot of electroosmotic velocity (how much flow is generated) against distance (in nanometers).

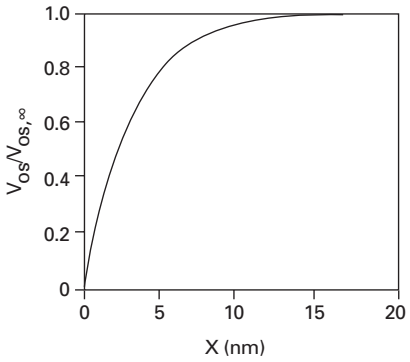

Figure 3. Calculated values of the electroosmotic velocity, V_{os}, as a function of the distance from the capillary wall, X, where $V_{os,\infty}$ is the fluid velocity in the bulk solution. From Churaev *et al.* (1981).

The normal water is being moved at a certain velocity because the skin is being pushed along by the electric field. There is a large amount of friction right at the wall; the liquid does not move because it is stuck to the surface. The velocity goes from 0 to the normal value for the flow very quickly (see Figure 3). Most of the water moves at a uniform velocity except the water right next to the edge, which is moving at a different velocity. This is called plug flow—a plug of liquid.

The plug flow allows uniform velocity over the entire capillary tube; it is negligible compared to the rest of the flow. The uniform flow rate is the primary reason peaks are so sharp in capillary electrophoresis. The plug flow causes everything to be in line and to

move at exactly the same velocity. All the analytes move down the capillary tube at exactly the same speed and do not deviate from each other, producing a plug flow—or flat profile—and a very narrow, uniform band. Narrow bands mean good separation.

In liquid chromatography, the liquid is driven by pressure created by the HPLC pump. Pressure causes a parabola to be formed and a parabolic flow profile (Figure 4). The center has a much higher velocity than the side; it does not reach the maximum velocity very quickly. Molecules are at different locations across the column and move at different velocities, so HPLC sample bands are quite broad.

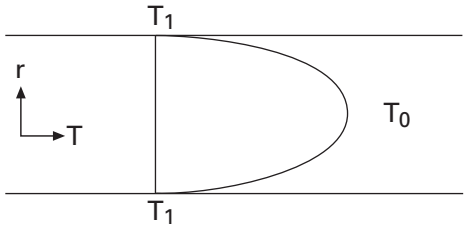

Figure 4. Parabolic temperature profile within the capillary. T_1 is the temperature at R_1, and T_0 is the temperature at the capillary center line.

Electroosmotic flow also changes with pH (Figure 5).

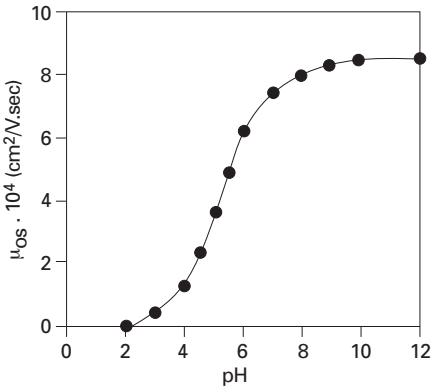

Figure 5. Dependence of electroosmotic mobility, μ_{os}, on buffer pH for the case of a fused-silica capillary that has been prewashed with 1.0 *M* NaOH. Reprinted with permission from Applied Biosystems (1990).

At low pH, there is almost no electroosmotic flow. At intermediate pH—pH 7.0 or so—electroosmotic flow is fairly high. When the pH is changed to separate weak acids and weak bases, the flow rate also is changing.

At extremely low pH, only electrophoresis is important—only the driving force of the electric field and the frictional retardation force determine the motion.

As pH increases, the separation becomes more complicated because there are three parameters: the driving force, the frictional force, and the electroosmotic flow. While changing one parameter, another may change.

At extremely high pH, flow will not change because it is already saturated. In that case, the result will also be reproducible.

Reducing electroosmotic flow is one of the ways to control a separation; it will be easier to fine-tune because there are two parameters to change rather than three.

To Reduce Electroosmotic Flow:

- reduce K^{-1}
- increase C_i (heating)
- decrease ε (organics)
- adsorb small cations
- covalently block silanol
- point of zero charge (pH < 2)
- increase η

Apparent mobility $\mu_{app} = \mu + \mu_{eo}$

Basically, all the changes to reduce electroosmotic flow are attempts to block the dissociation of the silanol groups. When blocked, there can be zero charge and, therefore, zero electroosmotic flow, even at high pH. The measurement is now apparent mobility.

Mobility is thought of as velocity. The apparent mobility that is measured is equal to the mobility determined from electrophoresis, ($\mu = q/f$)—the charge developed by the frictional force against it, plus the electroosmotic flow.

The apparent mobility is no longer unique; it depends on the column as well. This is difficult to understand when interpreting electropherograms because in liquid chromatography and gas chromatography, the flow rate is constant. There is a constant flow pump in LC and a gas cylinder produces a constant flow in GC, so the retention time or migration time is always the same.

In electrophoresis, it is constant due to the driving force and frictional force, but these change from one laboratory to another or from one experiment to another or from one capillary tube to another. These changes cause the peaks or the migration time to be irreproducible.

To understand electroosmotic flow, the observations can be separated into two parts: the part that is due to the electroosmotic flow and the part that is due to the driving force and frictional force.

To draw an analogy, it is like rowing a boat. Rowing is the driving force, which is the electrical charge. The boat has friction, water dragging the bottom of the boat, which is the frictional force, f. And the stream is moving by itself, so that is the electroosmotic flow. How fast the boat is going depends on the rowing, the friction, and the stream—whether rowing downstream with the flow or upstream against it. All these factors need consideration to calculate the net velocity or apparent mobility.

The last concept for electrophoresis is heating. Heat, even 10 W, changes viscosity. Things generally become less viscous as a function of temperature, just as a car tends to freeze up in the winter because the motor oil becomes thicker and thicker. Thicker and thicker means the viscosity is larger and larger.

Viscosity of water changes by about 2% for every 1 °C. In a laboratory, the temperature may fluctuate by 5° or so, changing the retention time and the velocity of the analyte, and that will lead to incorrect results in identification or in control. A 5° fluctuation is a 10% change, so in a 10-minute separation, the peak will move by 1 minute—completely unacceptable. In LC and GC, the velocities are determined by pressure, but in capillary electrophoresis, velocity is determined also by the temperature changing the viscosity, which is the frictional part.

Commercial instruments have built-in temperature controls. Some use liquid-heat exchange and others use air-heat exchange to stabilize the temperature, allowing reproducible results.

Current is passing through the capillary tube, so even at 10 W, a small amount of electrical energy is created. The electrical energy, called Joule energy or Joule heating, heats up the center of the capillary tube. The center of the capillary tube then has a higher temperature than the edge of the tube, and that affects the movement of the analytes.

Joule Heating
Density gradient leads to natural convection
μ depends on T (2% per °C)

While in capillary electrophoresis there usually is a flattop profile with everything moving in a uniform band, temperature differences can be observed (Figure 6). In actual experiments, the temperature at a side will be different from the temperature at the edge of the capillary tube. These differences create different velocities, depending on where the analyte is located, and that causes the band to become broad.

When measuring electrical conductivity, changes can be observed with respect to temperature.

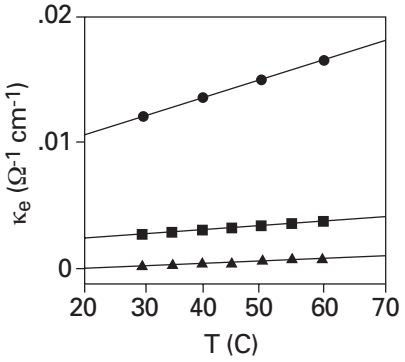

Figure 6. Measured values of the electrical conductivity, κ_e, of three different buffer systems: (•) 100 m*M* sodium phosphate, pH 7.0; (■) 50 m*M* sodium citrate, pH 2.5; (▲) 20 m*M* [cyclohexylamino] propanesulfonic acid (CAPS), pH 11.0. From *Capillary Electrophoresis. Theory & Practice,* P. D. Grossman and J. C. Colburn, eds., Academic Press, San Diego, 1992.

For More Information

The following textbook is recommended as an introduction to the basics of capillary electrophoresis and gives excellent examples for current uses.

Capillary Electrophoresis: Theory & Practice, edited by Paul D. Grossman and Joel C. Colburn. San Diego: Academic Press, 1992.

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Micellar Electrokinetic Chromatography (MEKC)

Developed about 1985, micellar electrokinetic chromatography is a modification of capillary electrophoresis so that neutral compounds can be separated. Before that time, only ions or ionic material could be separated, and in HPLC and in forensics, many things are not charged, and there was no way to use capillary electrophoresis. If neutral compounds and charged compounds can be separated, then basically every possible analyte is covered.

Electrokinetic means the whole system is driven by an electric field. The instrumentation used in micellar electrokinetic chromatography is exactly the same as in capillary electrophoresis. Chromatography means there is interaction with molecules: the analytes are interacting with a column. In MEKC, the analytes are interacting with micelles that are added inside a solution; there is no salt solution.

A micelle is a fancy term for detergent; most detergents are some form of micelles. Micelles allow a transition similar to how fat (e.g., an insoluble fat like butter) can be dissolved in water and then washed away in a two-step process. Micelles can be better controlled and are relatively inexpensive.

A micelle is composed of molecules that have ionic or polar ends and nonionic or hydrophobic ends. The molecule shown in Table 1 is $C_{12}H_{25}$. Basically, C_{12} is an alkyl straight chain carbon, just like paraffin. Paraffin is nothing more than a very long straight chain hydrocarbon; it is nonionic, hydrophobic, and will not dissolve in water.

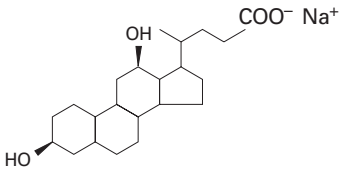
The other end of the molecule has a sulfonate group attached to this organic chain, so sulfonates are easily ionized and will be charged; that is the part that will dissolve in water because water likes ionic material.

Therefore, one end of this molecule is dissolved in water and the other end is not dissolved in water. That, in itself, is still not a micelle but just one component of the micelle. By mixing a lot of these molecules together, a 1–10 mmol concentration, a critical micelle concentration is reached; the molecules recognize the presence of other molecules and group together in a fuzzy ball.

Micellar Electrokinetic Chromatography (MEKC)

Micelles, critical micelle concentration (CMC), dynamic exchange, polydispersity T, buffer, organic content—

Table 1. Critical micelle concentration and aggregation number for some common surfactants used in MEKC.

Surfactant (abbreviation)	Structure	CMC (m*M*)[a]	N
Anionic surfactants			
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}OSO_{3-}Na^+$	8.1	62
Sodium octyl sulfate (SOS)	$C_8H_{17}OSO_{3-}Na^+$	136.0	20
Cationic surfactants			
Hexadecyltrimethylammonium chloride (CTAC)	$C_{16}H_{33}N^+(CH_3)_3Cl^-$	1.3	78
Dodecyltrimethylammonium bromide (DTAB)	$C_{12}H_{25}N^+(CH_3)_3Br^-$	15.0	50
Zwitterionic surfactant			
N-dodecylsultaine (SB-12)	$C_{12}H_{25}(CH_3)_2N^+CH_2CH_2CH_2SO_{3-}$	1.2	
Nonionic surfactant			
Polyoxyethylene-t-octylphenol (Triton X-100)	$(CH_3)_3CCH_2C(CH_3)_2C_6H_4(OCH_2CH_2)_{9.5}OH$	0.2	143
Bile salt			
Sodium deoxycholate (NaDC)		6.4	14

[a]Critical micelle concentration (CMC) and aggregation number (N) data reproduced with permission from Hinze (1987).

Association can be with
- hydrophobic core
- hydrophilic outside
- incorporation
- chiral

a "moving" stationary phase

Imagine that the ball is simply a group of molecules sticking toward the center. All the hydrophobic or the paraffinlike parts that do not like water, will go inside toward the center with all the ionic or polar groups sticking outside—like sticks assembled together. One ball of micelle will have 50 to 100 molecules, depending on the individual monomers or components. When there are enough micelle monomers, a ball is created that is polar outside and nonpolar inside. The organic molecules go inside because it is a favorable environment; they are hydrophobic and avoid water.

The outside is ionized, so the outside will be dissolved in water. The micelle dissolves the organic and then the micelle itself is carried out by water.

When a micelle is present, there is a moving stationary phase; a moving stationary phase is like the chromatographic packing inside a column.

In liquid chromatography, the column has beads, and the beads have parts where the organic molecules can adsorb, and that is why, by differences in adsorption, different compounds will come out earlier or later, depending on whether they stick harder or softer under the surface. That is the normal stationary phase.

The stationary phase is moving because the micelle is charged, so the micelle will be driven by the electric field. Even though it is moving, it is not that important; it is the center that is important. The center looks like the chromatographic column; organic molecules can go in there or be outside. The organic molecules on the outside move because of electroosmotic flow, not because the water is moving.

The organic molecules inside the micelle are moving with the charged micelle, which is driven faster because of the charge. The ionic molecules on the outside will move at a different velocity than the molecules on the inside of the micelle.

The key to micelle electrokinetic chromatography is different organic species will like the inside more or less. Molecules on the inside move at the micelle velocity; ionic molecules that avoid the micelle move at the electroosmotic flow. The difference in motion is important and allows separation.

The micelle ball will constantly have monomers leaving it and constantly have monomers reforming; it is a dynamic process. On the average, a micelle may have 50 molecules, but it could have 49, or 51, or 40, or 60. It is dynamically changing all the time.

Figure 7 illustrates the overall picture of electrophoresis. Interaction with monomers possible ion-pairing, ion-association, complexation—

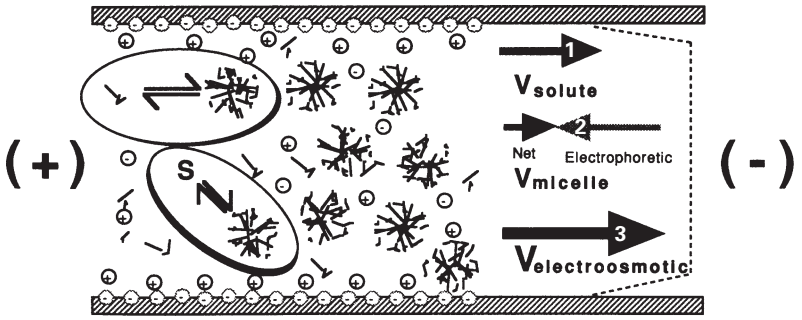


Figure 7. Depiction of flow dynamics in MEKC showing velocity vectors for electroosmotic flow ($V_{electroosmotic}$ or V_{eo}), micellar velocity [both its electrophoretic ($V_{e,m}$) and net (V_M) vectors], and solute velocity (V_{solute}). Also depicted are solute–micelle association and surfactant monomer–*N*-mer dynamics. Source: *Capillary Electrophoresis: Theory & Practice,* 1992, p. 164.

The capillary tube is shown; there is a charge at the walls because of the fused silica dissolving water and giving up its hydrogen ions, dissociating the silanol groups, and that results in a plug flow. All the water molecules are moving at constant velocity except very near the edge, and this whole sheath—this skin of the tube of water—is driven by the electric field moving everything in one direction.

The large arrow (arrow 3) shows the velocity of the electroosmotic flow, the water moving at its own velocity. Positive potential is applied on the one side so a positively charged analyte will move from the positive electrode to the negative electrode on the other side. The top arrow (arrow 1) represents q/f and could be pointed in the opposite direction if the opposite charge was applied. The arrows are drawn to show that the velocities are not all identical.

In zone electrophoresis, the sum of the top and bottom arrows (arrows 1 and 3)—representing the rowing boat and the moving stream—determines the time it takes to go down the column.

The micelles are represented as fuzzy balls with the organic content inside and the ionic content outside. The micelles move at a certain velocity, from right to left (arrow 2), because they are sulfonates, and negatively charged, they move away from the negative potential.

Inside the micelle, the net speed is the sum of all arrows 1, 2, and 3; outside the micelle, the net speed is the sum of arrows 1 and 3. The apparent mobility can be due to two interactions or it can be due to three: electroosmotic flow and normal electrophoresis or electroosmotic flow, normal electrophoresis, and micelle velocity.

As in liquid chromatography, there is the capacity factor, k′.

Limited elution range t_o/t_m
0.2 to 0.5 (= 0 for LC)
poor peak capacity, sees all neutrals

$$k' = \frac{t_R - t_o}{t_o\,(1 - t_R/t_m)}$$

R maximum for k′ between 1 and 5
(LC increases with k′), but can extend window if $v_{eo} = -v_{em}$

Change in SDS concentration above CMC does not affect v_{eo}

To reduce v_{eo}, lower pH or neutralize or derivatize

Different surfactants (cationic, neutral) for amines, phenols, bile salts, chiral

When k′ is zero, things move down the column without interacting. When k′ equals infinity, the solution is stuck on the column. Generally k′ is between zero and infinity in liquid chromatography. In capillary electrophoresis, k′ depends on how much time is spent inside the micelle and how much time is spent outside the micelle; just like in LC, how much time is spent on the column versus in the mobile phase.

The resolution is best if k′ is between 1 and 5, so it is important to adjust conditions; for example:

- **concentration—**a higher concentration of SDS, sodium dodecyl sulfate, increases the likelihood of molecules getting inside (see Table 1, p. 21).

- **pH—**the pH can be altered (i.e., neutralize or derivatize the material); pH changes the velocity of the molecule outside and also changes electroosmotic flow.

- **surfactants—**use different surfactants or different kinds of detergent, which can be cationic, positively charged micelles, or neutral micelles.

Micelles can be negative or positive and are very useful for separating amines, phenols, bile salts, and even chiral compounds (left-handed and right-handed molecules).

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Capillary Electrophoresis

2. Methods for DNA Sequencing and Genetic Analysis

Learning Objectives

At the end of this section, the viewer should be able to:

- Describe why DNA molecules cannot be separated with capillary zone electrophoresis and why the gel in capillary gel electrophoresis works to separate them.
- Describe the problems with using frigid crosslinked gels of the past and the benefits of using the newer entangled polymers.
- Describe the elements of a DNA sequencing instrument.
- Explain how capillary gel electrophoresis is used for genetic analysis (STR, DNA fingerprinting).
- Explain in detail how special entangled polymers are used to separate DNA.
- Evaluate electropherograms.
- State the target resolution and explain what it is telling us.
- Explain the optimal separation results and how you would achieve them.
- Recognize the importance of proper sample handling.
- Identify that a peak is DNA, not some other type of contaminant and why.
- Describe different ways of genotyping and the advantages and disadvantages of each.

- Recognize why it is almost impossible to deliberately spoil a DNA sample to give misidentification, making DNA fingerprinting acceptable.

- Describe the meaning of a homoduplex or heteroduplex result, how they assist in mutation detection, and identify them on an electropherogram.

- Explain the single point mutation process (SNP) and the advantages of using it over other types of genotyping.

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Introduction to Sieving Matrices in Capillary Electrophoresis

Capillary gel electrophoresis (CGE) is a rapidly developing method for separation of biopolymers. Initially, crosslinked rigid gels were used. More recently, introduction of entangled polymers has allowed easy filling and flushing of the capillary after each run without any significant loss of resolution. CGE has been used successfully for the separation of dsDNA, DNA sequencing fragments, proteins-SDS complexes, and polyamino acids.

The first impression when separating large molecules is that they are separated by size, and the fact that larger molecules move slower and smaller molecules move faster because of frictional force. DNA, however, does not function like that.

DNA is a polymer, and the DNA sequence has A (adenine), C (cytosine), T (thymine), G (guanine) with units of organic sugar and phosphates tagged on. When one more base or group is added to the DNA molecule, the DNA becomes larger.

Because a charge group is added with the phosphate groups, charge is added to the molecule. Mobility, $\mu = q/f$, remains constant for large DNA molecules.

The added charge almost exactly offsets the increase in size so DNA molecules, in general, cannot be separated by size. One DNA base can be separated from DNAs with two bases and those from three bases, but after 20 bases or so—every additional one added—the size and charge effect cancel out each other, and they all move at the same speed.

Electrophoresis in the slab format has been used to separate DNA for years because of the addition of sieving material (e.g., a process like sifting sugar and flour). Normal salt solution and micelles are not part of the process; only the sieving material is used. (See http://neo.pharm.hiroshima-u.ac.jp/ccab/2nd/mini_review/mr130/dolnik.html for an extensive description from one of the pioneers of sieving material.*)

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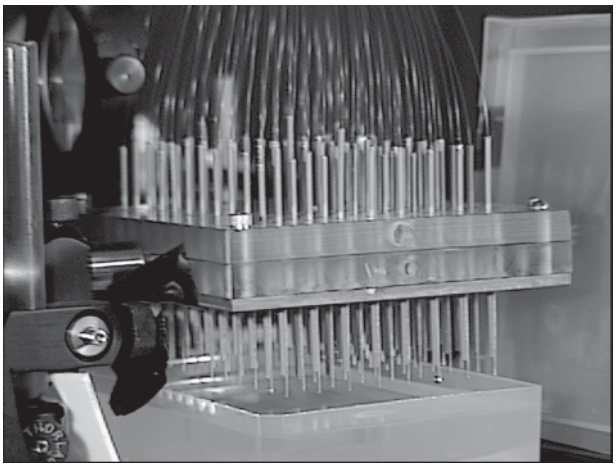
The sieving material is a gel, agarose—a common food additive. Agarose, at the right concentration, can separate DNA molecules. Useful in separating biopolymers, the gel is not exactly a liquid and not exactly a solid. Initially, gels were made rigid by crosslinking, a chemical reaction that forms a matrix and well-defined or rigid gel. Slab gels are polymerized to form the rigidity needed to separate molecules.

Recently, a process with entangled polymers allows the capillaries to be filled and then flushed so that the capillary tubes can be used repeatedly, minimizing both cost and labor. The solutions are used to separate all kinds of DNA molecules, proteins, polyamino acids, sugars, cellulose, and so forth.

Ninety-six capillary tubes are aligned with exact spacing in wells in an 8 × 12 (96) microtiter plate format that is commonly used in biochemical labs in robotics sample preparation. Each capillary fits in a well and has its own individual electrode so that 96 separations can be done at the same time.

Most HPLC and some GC instruments have a robotic interface, so that samples can run consecutively overnight. The computer selects one of the samples, injects it in the column, selects another, injects it in the column, and so on until all 96 samples are run.

Ninety-six samples can run at once because the entire plate is injected into all 96 capillaries simultaneously, and an electric field is applied that is common to all 96 capillaries.



Ninety-six capillaries are injected simultaneously.

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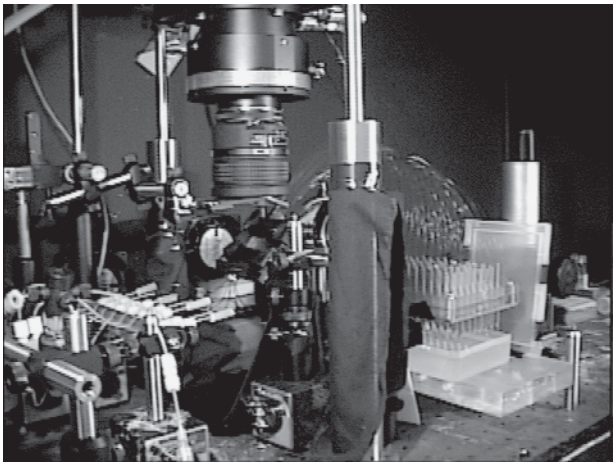
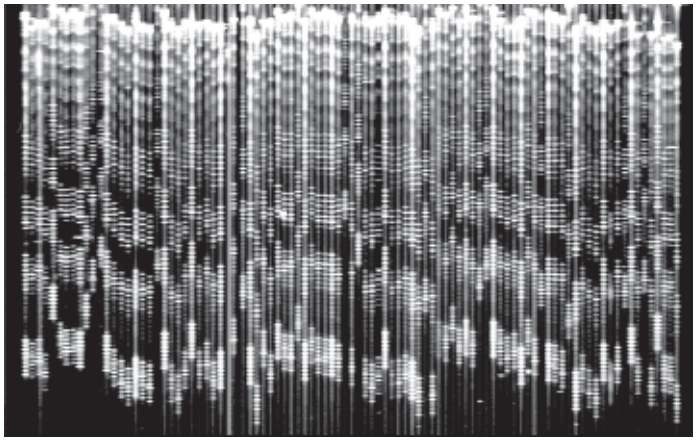
The laser beam scans the capillary windows, and the fluorescence from the molecules—the light emitted from the molecules—is gathered by camera lens into a CCD camera (a sensitive camcorder), and then all 96 capillaries are viewed on a screen at the same time. This technology allowed the human genome to be sequenced in a short time; DNA sequencing was the driving force behind its development.



DNA sequencing instrument.

The human genome consists of three billion (3×10^9) bases of alphabets that had to be read 96 samples at a time, and each sample had 500 to 600 bases. The sequencing was completed in a day-and-night factory-type operation in a relatively short period of time.

In forensics, short tandem repeat analysis (STR), shown below, is one way to produce unique DNA fingerprints from a sample.



Ninety-six capillaries, each with DNA; DNA bands light up when the laser light excites them, and they fluoresce. Reconstructed from computer data.

In slab gel, each capillary is recorded by one part of the camera and converted by the computer to light and dark areas and redisplayed in that format. Ninety-six independent experiments run with different types of samples.

Figure 8 shows four different genetic regions of human DNA.

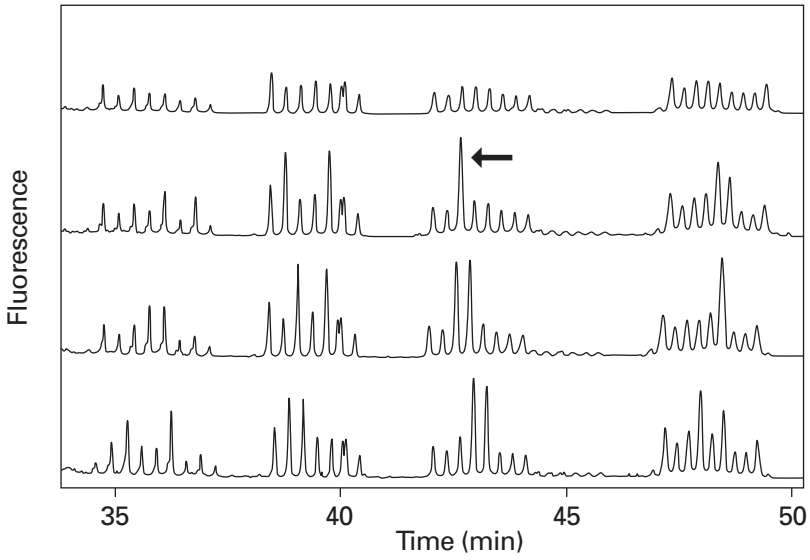


Figure 8. Four different genetic regions of human DNA. The top row is a standard sample and the bottom three are different unknown samples. Raised peaks (e.g., arrow) indicate the unknown sample has that particular gene.

In each region, each of our genes can have one or two bands; the standard sample (top row) contains all possible combinations of human genetic materials, basically a pool of many individual samples over different races, countries, sexes, etc.

There is a possibility of having eight different kinds of DNA links, depending on your gene—one if you inherit the same gene from both of your parents, but two bands if you inherit different genes from different parents.

In the unknown samples, particular peaks are raised or have high intensity compared with the standard, indicating that an individual has that particular gene. Finding these differences is DNA typing.

An accepted standard has between 13 to 15 regions, and for each region, there could be one or two possibilities. The possibility of matching or not matching a particular pattern is about one chance in 250 million—roughly the population of the United States—so a single individual could be identified in such a pool.

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Although legally DNA fingerprints are used to *exclude* individuals, technology has made it possible to use DNA to *identify* individuals as well. The chances of misidentification are negligible when many genetic regions are screened at the same time; DNA separations are widely accepted for forensic applications.

The DNA molecules are of different lengths—one is four bases longer than another, another is four bases longer than that, and so forth. The number of samples and the number of individuals make it necessary to separate at a high rate; the most efficient method is by multiplex capillary electrophoresis so that many samples can be done at one time.

A crime scene may have multiple bloodstains and multiple tissue samples or biological fluids to sample. Each sample might have a different individual as an origin. To screen that using 96 wells at the same time, all the samples could be analyzed in 20 to 30 minutes.

Why Multiplexed Capillary Electrophoresis?

- Standard chemistry and separation mode
- High resolution because of aspect ratio and low Joule heating
- High speed because of high applied voltage
- 150 micron o.d. (outer diameter) = 15 cm for 1,000 channels
- 10 microamp each = 10 mA for 1,000 channels
- 10 nL samples = potential cost reduction in sample preparation, labels, enzymes
- Less waste disposal
- Suitability for automation
- Standard microtiter plate format

Capillary electrophoresis copies the separation method and handling of DNA from slab gel electrophoresis, except in capillary electrophoresis, the resolution is four times higher. Higher voltage means higher speed, so the answers are obtained faster.

Thinner capillary tubes are 150 μm in diameter; 1,000 would require only 15 cm of space. The largest instrument currently available holds 384 capillaries (a multiple of 96), but the technology is there to build a larger instrument.

The current required for operation is tolerable; 10 mA of current means that the equivalent of a car battery can operate one of these instruments. Eventually, a van could be used as a mobile laboratory at the crime scene.

Only 10 nL of sample is needed—a small amount of material. Fewer biochemicals are needed to treat the DNA and that means less waste disposal.

The 384-capillary instrument that is commercially available is automated, completely computer-controlled, and can be run without supervision. Modified from the standard 96-well microtiter plate format, 384 microtiter plates are available. Pharmaceutical companies actually use 1,536 microtiter plates (4 × 384) by drilling smaller holes and then packing in the same area for multiple analysis.

New Separation Matrix

- Poly(ethylene oxide) or polyvinylpyrroli-done in TBE and urea
- Reproducible and easily prepared from commercial polymer powder
- Stable for months
- Usable with uncoated columns
- Low viscosity to simplify fluidics
- Cheap—20¢/g; i.e., 1,000 capillaries
- Nontoxic—similar to food additive
- Polymer mixtures for optimal separation over a large size range
- Can optimize for fast separation or long reads

The technology of DNA sequencing and genetic analysis depends on the separating material and that is different from the free solution used in CZE or the micelles used in MEKC. The separating material is a polymer—a polymeric material, such as polyethylene oxide or polyvinylpyrroli-done in TBE (borate buffer with EDTA) and urea. Borate buffer and urea are used in slab gel.

Instead of using polyacrylamide that is used in normal slab gels, other polymers were invented that are easier to handle. The polymers, a long chain material, are somewhat soluble in water—not highly soluble. The individual group is ethylene oxide, so there is some oxygen, and there is some polar interaction; that is why these polymers are water soluble.

Polyethylene oxide and polyethylene glycol are used in certain food additives to add thickness. Not originally intended for capillaries, polyethylene oxide remains stable for months and is nontoxic and environmentally friendly.

The polymers have low viscosity, which means that they are not very thick; the liquid can be pushed inside the column and pushed back out. Because of the small diameter, thick material would create flow resistance and if the material is too thick, it cannot be replenished run after run.

The polymers cost about 20¢/g; 1 g of material is needed for 1,000 capillaries—extremely low cost.

Capillary gel electrophoresis is performed in a capillary filled with a network of obstacles, most frequently crosslinked gels or entangled linear polymers. The space between obstacles forms pores through which the molecules of a polyelectrolyte migrate. The electromigration through pores reduces migration velocity proportionally to the size of migrating molecule and reciprocally proportionally to the pore size. Depending on a relative size of a molecule of polyelectrolyte and a pore, three principal modes are generally recognized: Ogston sieving, reptation without stretching (orientation), and reptation with stretching (orientation).

When polymers are dissolved, they run into each other, creating obstacles and forming an obstacle course that DNA has to move through. Large DNA molecules are separated from small DNA molecules because the large molecules slow down while the small molecules sneak through. There are different models of polymer separation, but Ogston is the sieving model. When it is a finer sieve, small molecules are favored; with a coarser sieve, large molecules also will flow through.

Other mechanisms are reptations (so named because of their reptilelike nature); DNA molecules can uncoil and form long chains, like a snake. In this snakelike form, molecules can swim through the polymer network and sneak through.

Theory of Sieving Matrices in Capillary Electrophoresis

Figure 9 illustrates how polyethylene oxide or polyvinylpyrrolidone, the commercial polymers, coil like string. Because they are polymers, they are a straight chain but coil up randomly.

If the solution is very diluted, each polymer will act like its own big molecule, one clump of string, and they will not run into each other, $C < C^*$ (Figure 9A).

At higher concentration, the volume that is necessary for the molecules to exist individually changes, and they begin to attach or come in contact with each other and form a group of polymers, $C = C^*$ (Figure 9B).

At even higher concentrations, there is not enough volume, and the stringlike polymers have to run into each other's space and entangle. It is at this point that a mesh is formed, like a sieve or screen, and selected molecules can flow through. If the molecules are small in size, they will sneak through, or if they are large, they cannot sneak through as easily, $C > C^*$ (Figure 9C).

$C = C^*$ is the critical concentration, where the molecules begin to run into each other and the sieving network begins to form (Figure 9B).

The screen is dynamic because the strings are moving around each other and the mesh is changing all the time. There are no separations until the screen is formed and DNA molecules can move through.

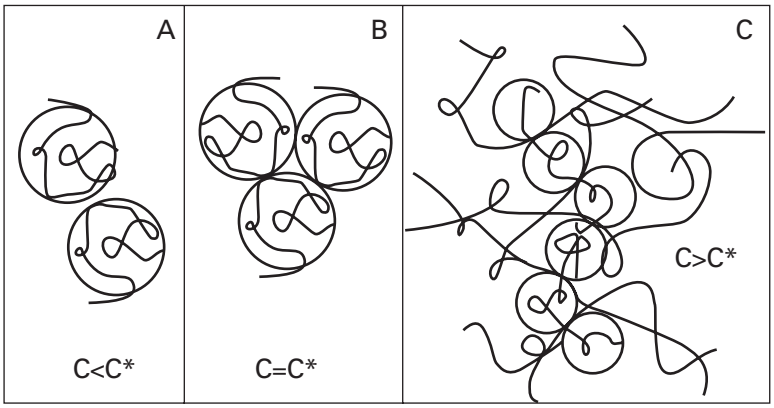


Figure 9. Schematic representation of flexible polymers in solution (A) diluted solution, (B) solution at entanglement threshold C*, (C) semi-dilute solution. The small circles in (C) represent the "blobs" of size ξ_g.

Optimizing Separations in Sieving Matrices

Figure 10 shows a sample of DNA molecules of different sizes.

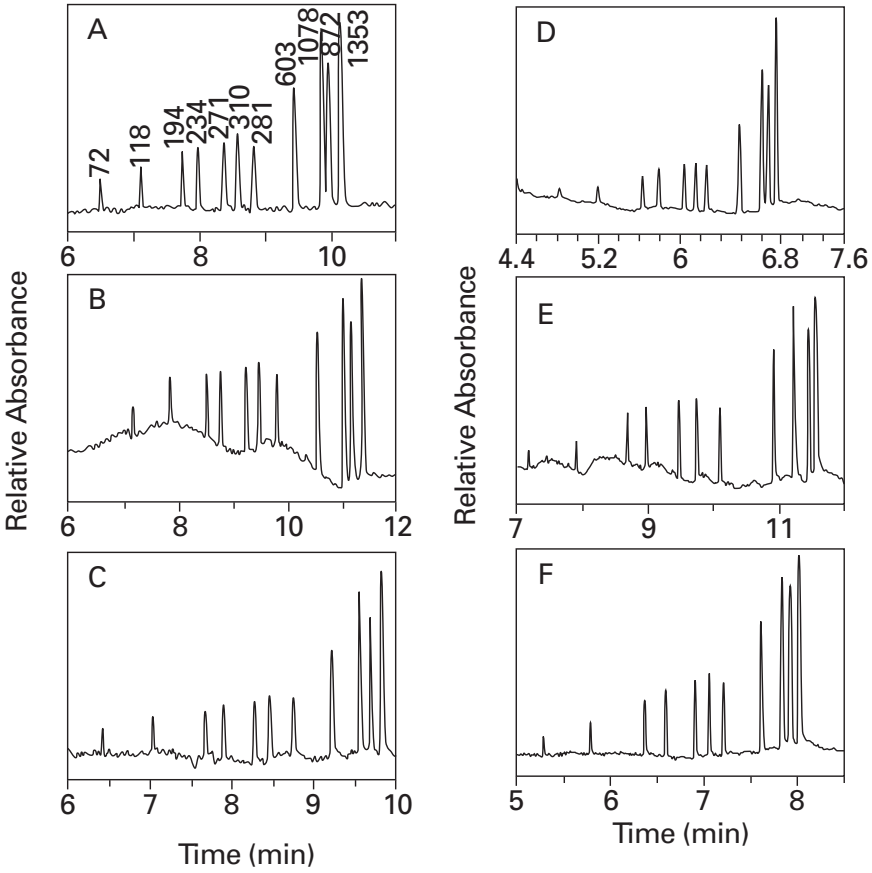


Figure 10. DNA molecules of different sizes.

The numbers (72, 118, 194 . . .) indicate the number of bases linked to the molecule. A base will consist of a sugar-phosphate group plus the DNA base, which is an aromatic group like purine and pyrimidine.

The DNA molecule is larger as the number increases. The smaller molecules move faster, come out faster, and the large molecules stay behind. Figure 10 is a DNA separation in polymer solution and looks like one found in slab gel. The difference is that the bands are much sharper than in slab gel; there is resolution improvement, and the resolution needs to be as high as possible.

When the concentration of the polymer changes, the peaks become sharper Figure 10B). There are more peaks in between, and more peaks mean that a DNA fragment can be identified with more certainty.

The series of experiments (Figure 10A–F) show the effect of changing the concentration and the property. All of the peaks are sharp at the top, but at the bottom, some are much narrower than others. The peaks (Figure 10F) are extremely sharp, showing that by changing the concentration and how it entangles, the separation can be changed.

Electropherograms can be evaluated by plotting the resolution per base pair (Figure 11). A base pair is one more DNA unit, so between 72 and 112, there are 40 base pairs. The distance of separation between pairs, divided by 40, is the resolution per base pair.

Figure 11 shows the six experiments, and each data point corresponds to one of those peaks; a different performance for separation can be figured for each DNA fragment.

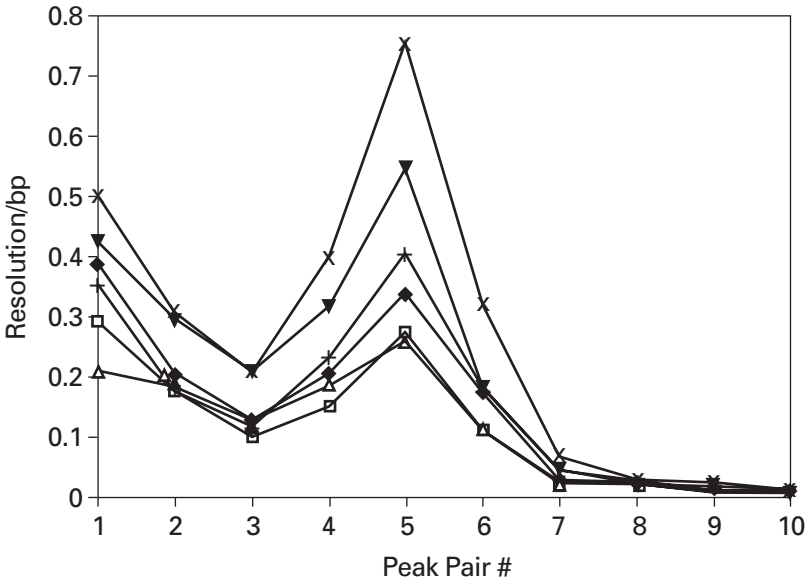


Figure 11. Resolution per base pair.

The desired resolution is 0.5 because that means two DNA molecules that are different from one base—adding one more base to it—will be separated and distinguishable. Half a DNA base cannot be added. A resolution of 0.5 theoretically allows one absolute confidence in determining the molecular weight of the DNA.

In Figure 11, the middle region shows good polymer performance because it is above 0.5, but this polymer is not good enough to separate the smaller molecules or the larger molecules

with that same degree of certainty. This is used as a criterion for DNA sequencing because DNA sequencing counts every DNA base—one must see every base.

For the forensic application of STR, short tandem repeat analysis (see page 30), the peaks are separated by four bases. That is a resolution of 0.125; each is 0.125, so four bases will be 0.5 and successive peaks will be resolved. Therefore, Figure 11 shows that, at 0.125, some of the peaks will work for forensic applications, some are still below par, and some are unacceptable.

Such experiments are useful to demonstrate that the separation system is good and to give credibility in saying this DNA belongs to this particular genetic origin.

Figure 12A shows a poor separation with about 20 different DNA fragments. In one type of polymer composition, the peaks on the right, fragments 16–20, are well separated, but the others are not well separated and bunch together, giving unresolved peaks.

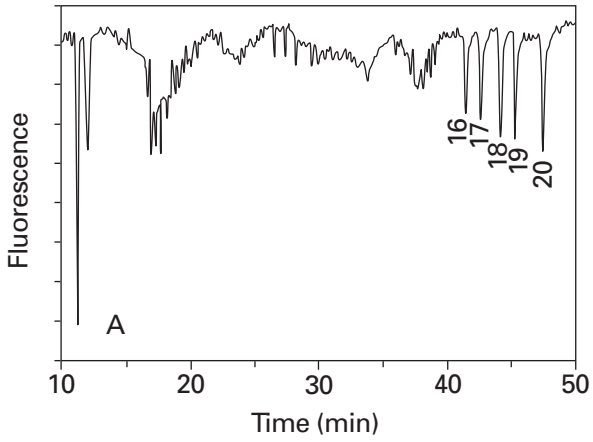


Figure 12A. Comparison of resolved and unresolved peaks.

Some polymers separate large DNAs well. Other polymers, if fine-tuned, can separate over a wide range of DNAs. If done properly, small DNAs as well as large DNAs are all well resolved and that is a critical test to validate a DNA separation.

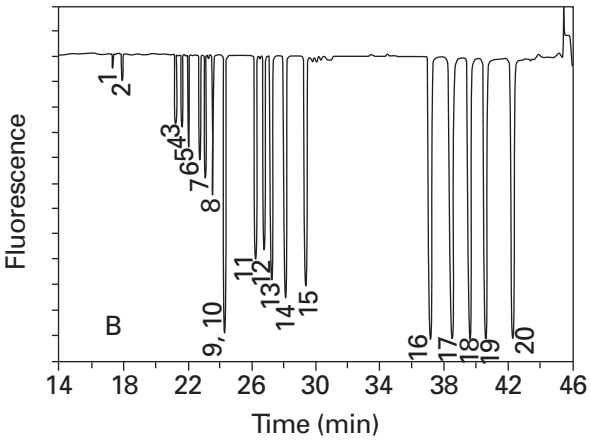


Figure 12B. Example of resolved peaks, but the experiment still fails.

Note that one peak actually contains two fragments: 9 and 10. They are not resolved because they are superimposed upon each other and because of these two particular fragments, this plot fails.

Figures 12A–F show the attempts to resolve peaks and gradual improvement. Figure 12E shows the two fragments are finally split into two distinct peaks, and Figure 12F shows the best separation with all peaks resolved. This experiment demonstrates that, by changing the polymer material, good separation can be achieved.

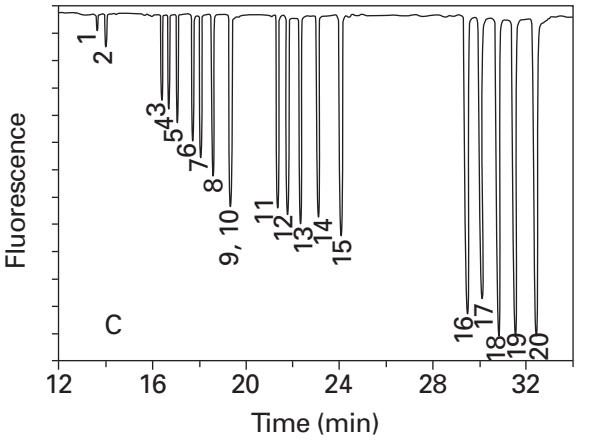


Figure 12C. The plot shows continued improvement.

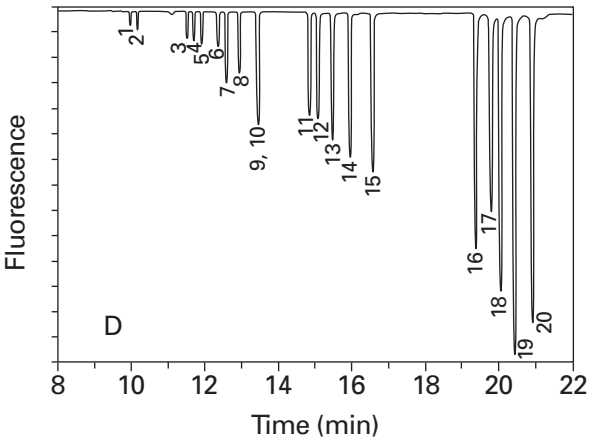


Figure 12D. The plot shows continued improvement but needs fine-tuning.

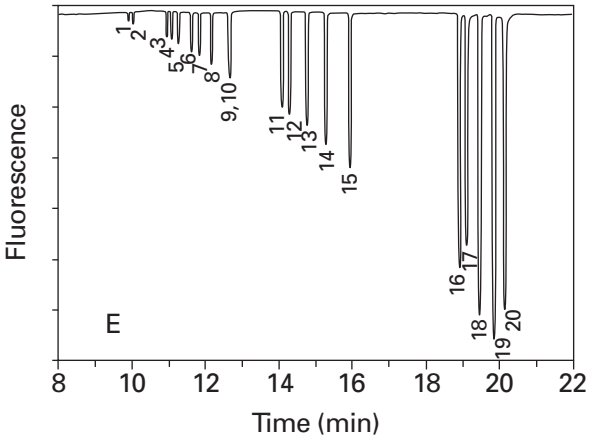


Figure 12E. The two fragments become two distinct peaks.

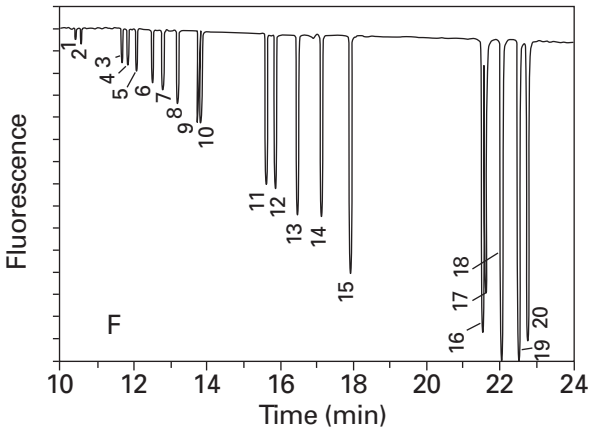


Figure 12F. All peaks are resolved.

Figure 13A and B are plots of the previous set of data. The top trace is always above 0.5, indicating good separation material for a DNA sequencing application, not just STR analysis.

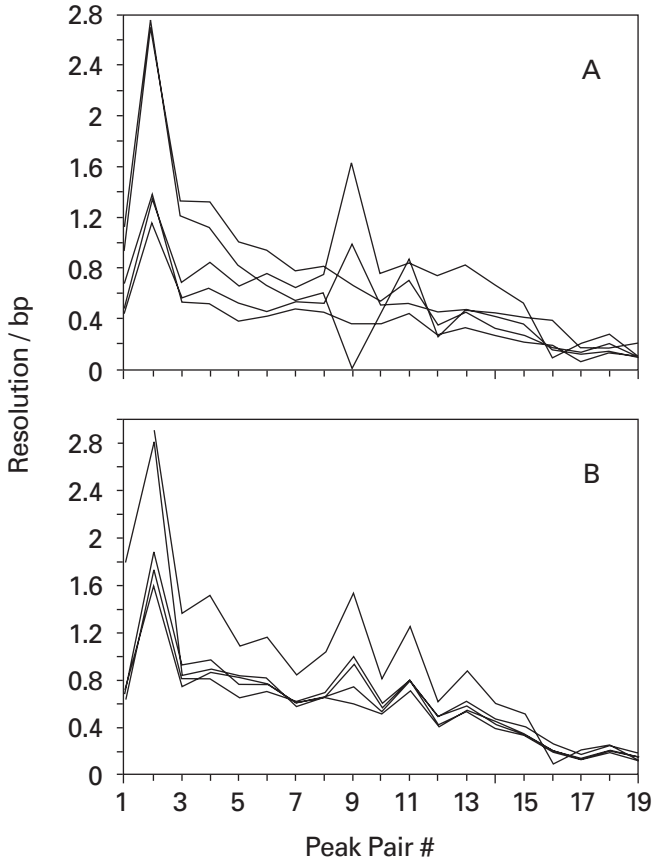


Figure 13. Plots showing the top trace above 0.5, indicating good separation material for DNA sequencing.

The materials are all commercialized so it is not necessary to develop a separation for DNA; the chemical properties and migration properties are all the same.

Conditions need to be adjusted to separate proteins by size. Some proteins are flexible, some more rigid. Even though molecules are the same size, they may not separate in the same way.

DNA matrices of polymer solutions can be purchased in bottles, put into the machine, and looked at as any other buffer system.

Another problem with DNA separation is the coated column. Remember that the column itself is negatively charged, and whatever is placed in the capillary tube moves with the electroosmotic flow. In the early days of DNA sequencing, when the electric field was applied, the gel inside the tube—all the stringlike material—was ejected from the capillary tube because it was pushed out by the electroosmotic flow. That was not acceptable, so the column was then coated with an organic material, like a polymer, to permanently shield the charges on the capillary. When the electroosmotic flow is stopped, the material remains stable, the gel stays in, and only the DNA molecules move through the sieving material.

Figure 14 shows different DNA fragments for DNA sequencing and the separations from two coated columns, one with polyethylene oxide (PEO) and the other with polyacrylamide (PA), a traditional material for slab gel electrophoresis. The separations look about the same, not particularly interesting.

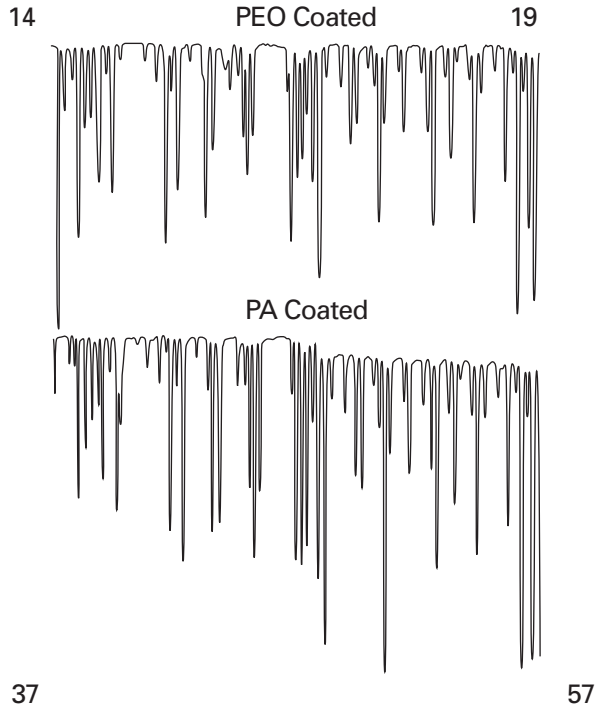


Figure 14. DNA fragments separated with two different polymers, polyethylene oxide (PEO) and polyacrylamide (PA), used to coat the column.

Figure 15 shows a continuation of the previous trace in terms of time, but the molecules are larger. The polyethylene oxide has better resolution—the peaks are broader with polyacrylamide—so polyethylene oxide is a better material even though polyacrylamide is more commonly used for separation.

Examine the entire separation, not just one region. If small DNA is of interest, then a lot of polymer material will work. If larger DNA fragments are targeted, getting more information out of the samples, then one must be highly selective on the sample.

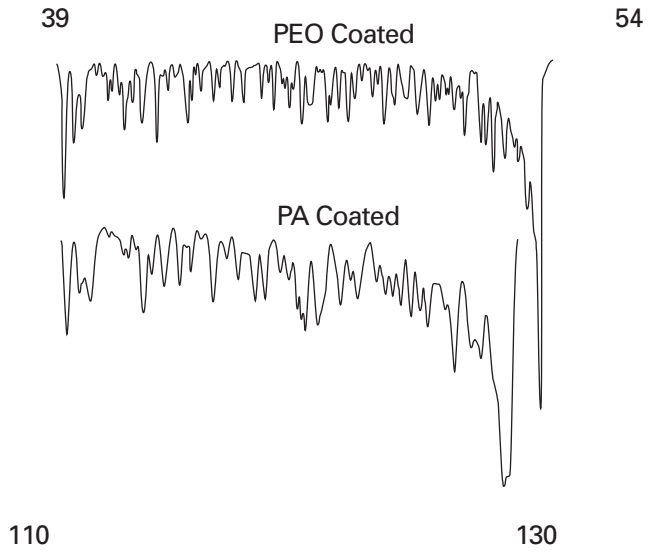


Figure 15. As DNA molecules become larger, the differences in peaks between polyethylene oxide (PEO) and polyacrylamide (PA) show that polyethylene oxide gives better resolution.

In uncoated columns when the surface charge is unshielded, the polyacrylamide material will be extruded from the column when the electric field is applied. There will be no electropherogram because the gel is pushed out; there is no screening effect inside the capillary tube.

Polyethylene oxide, however, is interesting because polyethylene oxide itself serves as a coating on the column. Without taking the extra step to put a polymer on the column, polyethylene oxide molecules will distribute on the surface of the column to shield the charges. With polyethylene oxide, the result is the same in either a coated or uncoated column (see Figure 16).

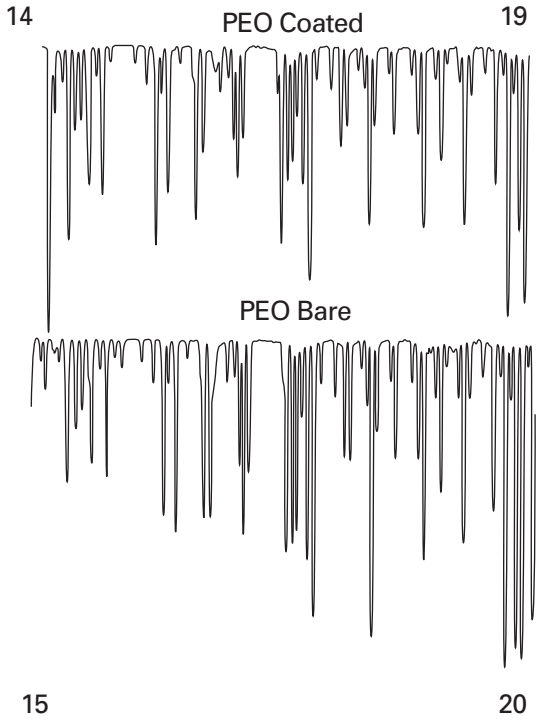


Figure 16. Polyethylene oxide gives the same results in a coated or uncoated column. Note the 5-minute time lapse.

Many peaks of DNA separated in a short period of time because a high electric field was applied (Figure 16). When the electropherogram is expanded (Figure 17A–E), there are individual sharp peaks at extremely high resolution (E).

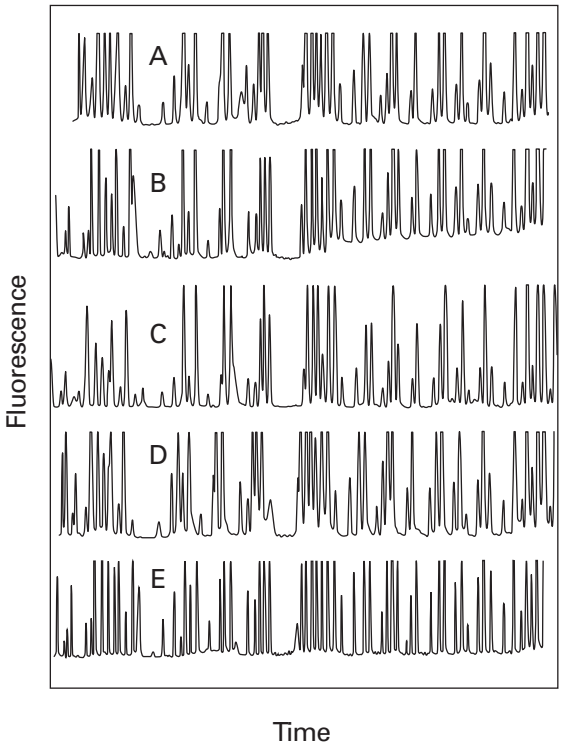


Figure 17A–E. An expanded electropherogram showing high resolution.

Higher resolution and sharp peaks are possible in gas chromatography. In liquid chromatography, it is not possible; the bands broaden because of the shape of the parabolic flow and chromatographic retention differences.

A count of theoretical plates is used as an indicator of performance. In liquid chromatography, there are probably 3,000 to 4,000 theoretical plates on a column, and that is considered very good. In gas chromatography, there may be a few thousand theoretical plates, but in capillary electrophoresis, there may be two million theoretical plates. The peaks are extremely sharp, allowing complete baseline separation of one DNA from another DNA that is only one base larger.

Figure 18A–E is a continuation of Figure 17A–E with differences made by changing solution mixtures. This example shows that more or less information can be obtained from the same electropherogram.

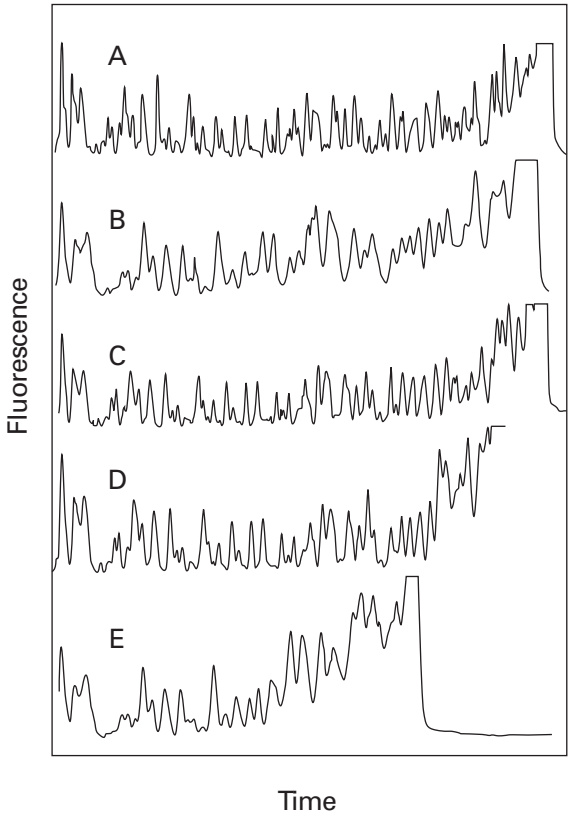


Figure 18A–E. A continuation of the electropherogram with differences made by changing solution mixtures.

NOTES

Figure 19 is an example of DNA separation and DNA sequencing. The computer analyzes the peaks, notes the color, and calls the base, deciding A, C, T, G, and so forth, from the separation. Four hundred DNA fragments were separated from each other within a time span of approximately 6 minutes.

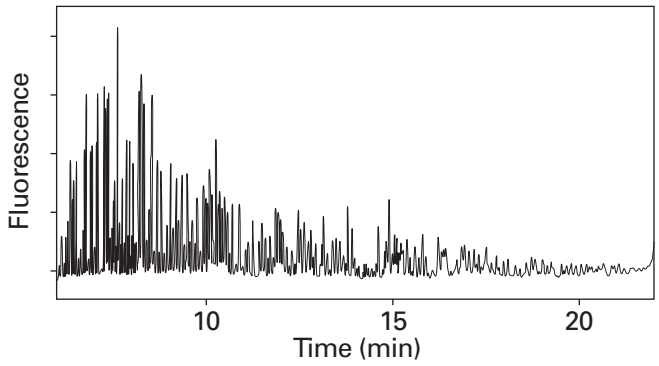


Figure 19. High-speed DNA sequencing by CE. The primer appears at 6 minutes and base 420 appears at 16 minutes. The average rate is 40 bp/min.

Genotyping or Genetic Analysis

Figure 20a–c shows the DS180 region of human DNA called double-stranded DNA (dsDNA). A particular person may have one or two peaks in this region that contains approximately 20 peaks.

In the right-hand area of Figure 20a and 20b, note that the separations are not producing two fragments at the end; the condition shown in Figure 20c finally separates the two. A lot of effort is spent in situations like this because the DNA sequencing gel is normalized for sequencing DNA, but it is not optimized for separating other genetic regions. For DNA typing—genotyping—it is necessary to buy separate polymers or mix a separation matrix to separate such high molecular weights. Figure 20a shows up to 800 base pairs; normal DNA sequencing is only concerned with fragments up to 500 or 600 so reoptimization is needed.

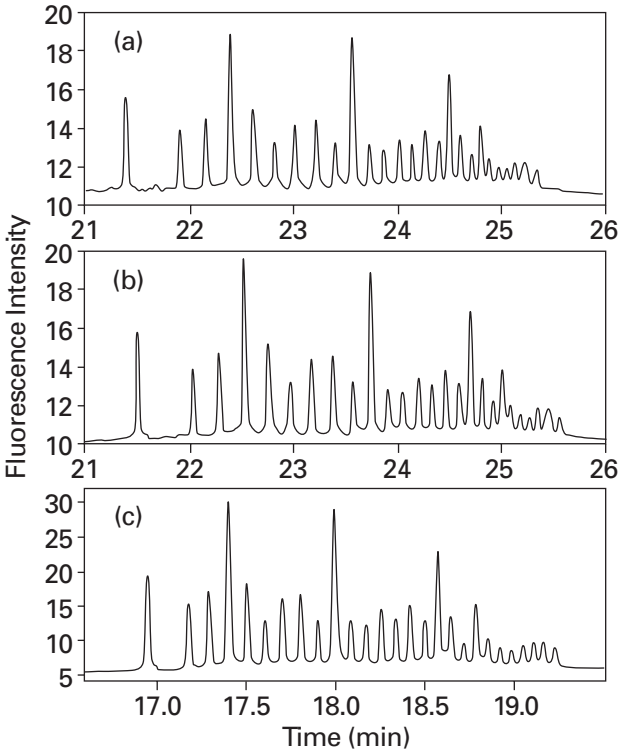


Figure 20a–c. An example of attempts to separate two fragments in the DS180 region of human DNA, called double-stranded DNA (dsDNA).

Figure 21 is an example for genotyping two fragments, 212 and 218, amplified only in the Y chromosome, an indication of whether the sample is from a male or female. In this case, it is six base pairs apart and can be separated very easily.

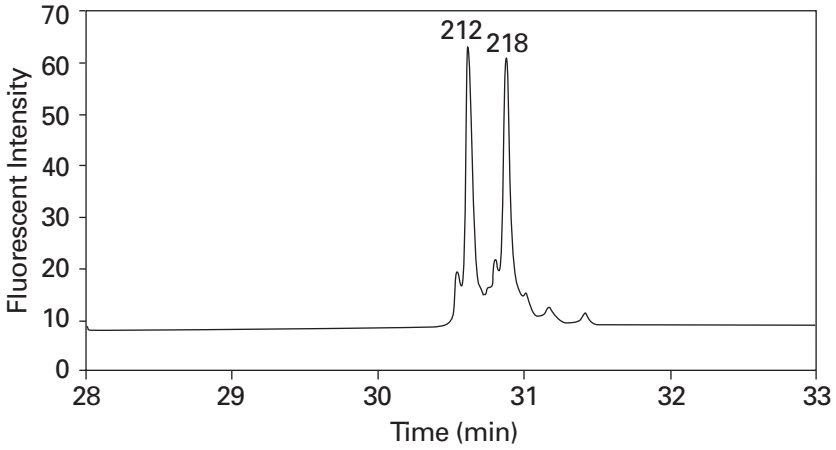


Figure 21. Genotyping two fragments, 212 and 218.

There are lots of applications for genetic analysis, but there are some problems with the current set of genetic measurements. Figure 22a–c is the same genotyping, short tandem repeat analysis, found in Figure 20, except there are additional sets of peaks (asterisks) that show up in this region (Figure 22b).

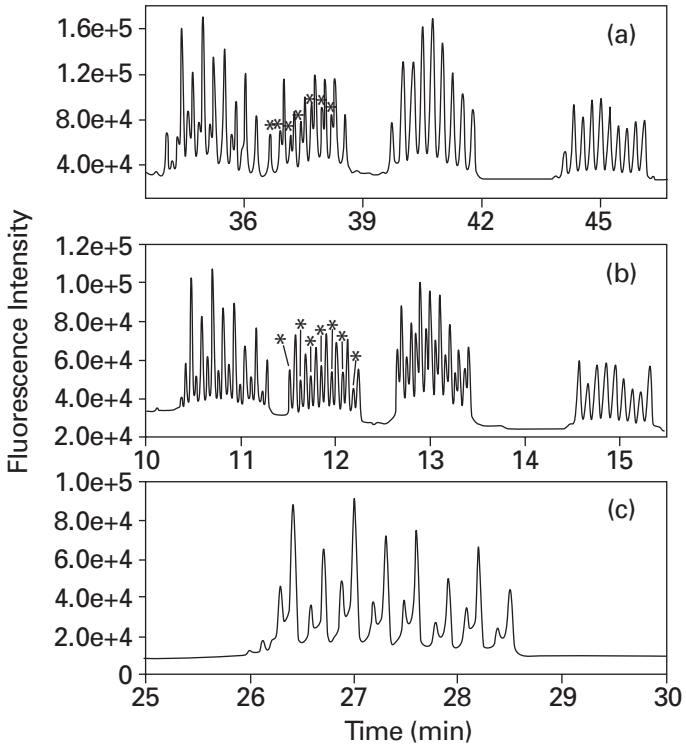


Figure 22a–c. Genotyping showing additional peaks caused by improper sample preparation.

Expanded in Figure 22c, there are clearly two sets of peaks, not just one. An error was introduced in sample preparation, so each correct fragment is now showing a side peak. In this situation, there cannot be confident identification of the sample and this is not acceptable for DNA fingerprinting.

DNA fingerprinting involves good separation but also good sample handling and sample preparation procedures. Artificial peaks must be eliminated so that there is only one clean set of peaks to measure and identify.

DNA peaks can be distinguished from peaks that are created by contaminants in the environment such as proteins or other small molecules. DNA usually will not give fluorescence until dyes are added to the solution. The dyes are very specific and only bind

to DNA because DNA is a double helix—basically like two strings wound together—and the dyes lie in between the grooves of the DNA so they intercalate or adsorb under the grooves of the DNA.

A few proteins show this kind of adsorption, but almost every other kind of molecule will not bind to the dyes and will not show any signal.

DNA molecules are very large—112 base pairs or so. One hundred base pairs times 300 or 400 MW per base pair is a very large molecule. Normally, environmental contaminants—dust particles, chemical agents—will be much smaller than that; they will not be in the region one is trying to probe.

Furthermore, the contaminant must bind exactly to the dye, and there are not many known compounds that will bind exactly to the dye.

To deliberately spoil a DNA sample or to cause misidentification, one would have to create an exact DNA fragment similar to what is in the genetic code, exactly the same size, and add it to the sample in exactly the right amount because all the normal genetic material is there.

A DNA fingerprint band observed in capillary gel electrophoresis is extremely unlikely to come from any normal environmental contaminant and is with a high degree of certainty DNA. If the band matches the band from the DNA sample, dust came from the sample. It is extremely unlikely that someone knew in advance what fragment the sample DNA contained, synthesized that fragment in a lab, and then dumped it into the sample.

If lab technicians' DNA got into the pool of suspects' DNA, additional bands that correspond to the genetic code of every individual whose DNA is in the mixture would appear and could be excluded.

DNA fingerprinting is very powerful, extremely difficult to contaminate, and that is why it is acceptable as genetic evidence.

Figure 23 shows an example of spiking, an experiment to confirm the presence or absence of a DNA band.

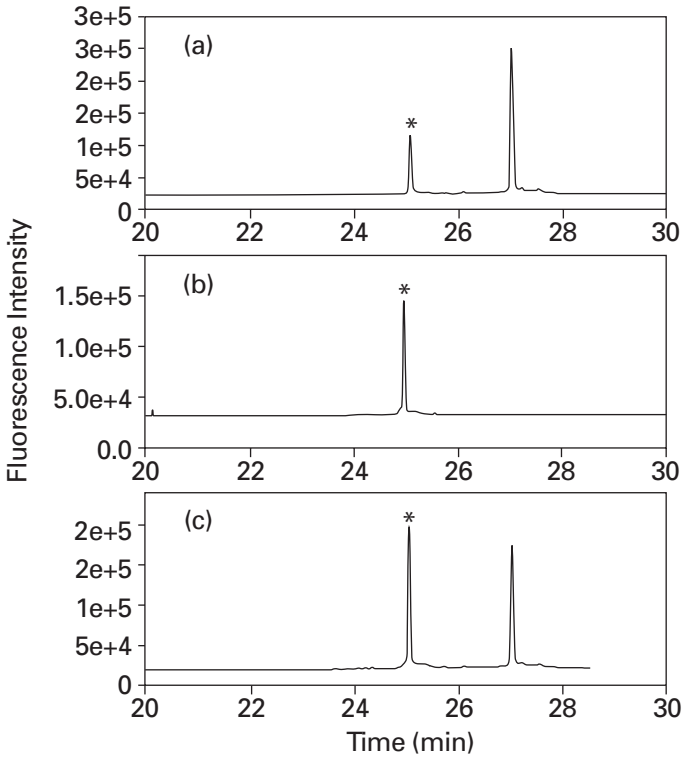


Figure 23a–c. A three-step spiking technique used to confirm DNA.

Figure 23 shows the standard (a) and the unknown suspect under investigation (b). It would be difficult to convince a jury or the court that the two asterisked bands are identical because of slight differences in exact time; they do not look exactly the same—one of the common problems using slab gel. The spiking technique (Figure 28c) then mixes the sample and unknown together to prove there is no experimental error and the two DNAs are identical.

High resolution makes DNA fingerprinting more difficult. Figure 24 shows peaks four bases apart, except in the THO1 region of the human genetic code where one genetic trait misses one of the bases. DNA sequencing instruments will always need to be performing at the maximum resolution for all peaks to be useful.

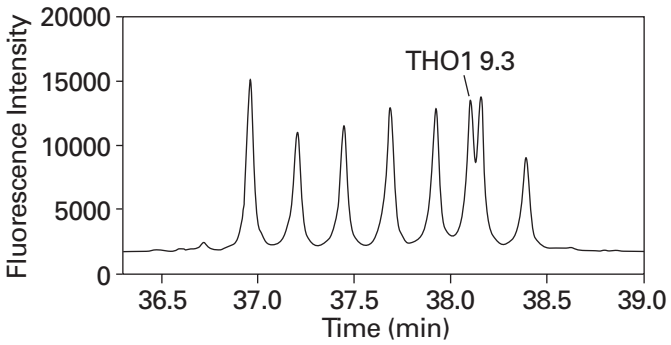


Figure 24. An example of DNA fingerprinting, four bases apart, showing the THO1 region of human genetic code missing one of the bases.

Figure 25a–c shows a new temperature-dependent polymer developed for DNA separation at 20, 71, and 74° C. The differences shown are significant; good separation for a DNA fragment occurs only at 71° C.

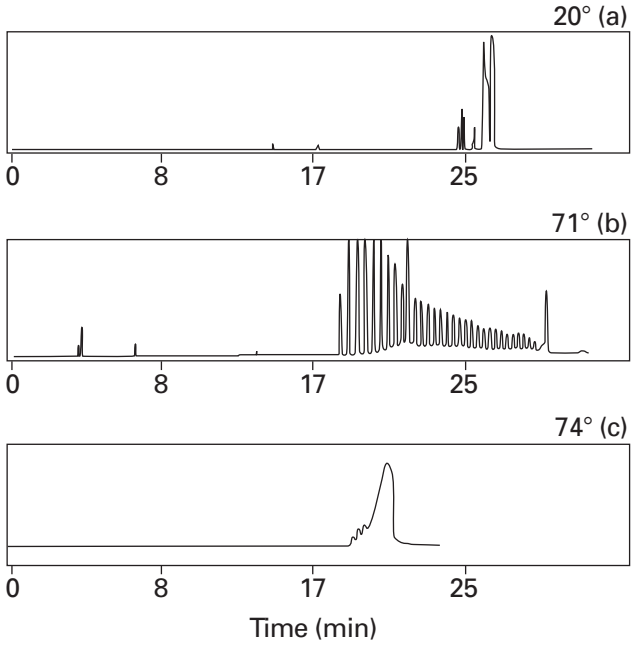


Figure 25. An example of how a temperature-dependent polymer affects separation at 20, 71, and 74° C.

In order for DNA separation to occur, the polymer strings have to form the mesh. At low temperature, the polymer has small volume, and the strings do not run into each other (Figure 25a). As the temperature increases, the polymer swells; as it swells, DNA separation occurs (b). At high temperature, the polymer swells so much that it cannot hold itself in solution; it is no longer a polymer and precipitates out.

At low temperature, the solution looks like water and is very nonviscous. It does not separate DNA, but handling is easy: it can be pushed in a capillary tube and can be poured from one bottle to another.

When in actual use, the instrument is set at 71° C to form the mesh and do the separation; then the column is cooled down, the polymer becomes waterlike, and can be flushed out and replaced. The instrument's design and the operation of DNA sequencing is much easier and faster with temperature-dependent polymers.

Many polymers are temperature dependent (e.g., the liquid crystal display on a watch is like a temperature-sensitive polymer. It forms a liquid crystal face at a certain temperature. Little heaters heat up the elements, and it looks dark because of the transition between a disordered and ordered polymer).

In the future, DNA sequencing instruments will become more user-friendly. Temperature-dependent polymers will prevent clogs and prolong the life of the column.

Mutation Detection in Sieving Matrices

Single-point mutation is not currently used for forensics applications but has the potential for eventually adding confidence to DNA fingerprinting as a forensic tool. The technique is a simple, fast, and reliable way to determine single-base differences. It is forensically important because one is comparing an unknown sample with a known individual, and it is only necessary to determine *if* it differs, not *where* it differs.

For example, a huge 600-base DNA molecule is identical everywhere except one base—one very small part—is different. Figure 26 illustrates the technique with a C-G double strand DNA (C always pairs with G). In the mutant, C is replaced with T, a T-A pairing (T always pairs with A). If the two were put into a capillary, they would probably come out at exactly the same time and could not be distinguished.

In mutation detection, the standard or normal DNA is mixed with the DNA under investigation—the suspect's DNA. When heated, the DNA strands, which are bound together by hydrogen bonds, will move apart, actually dissociate from each other, and form four distinct single strands.

When the sample is cooled, the single strands will match pairs again, but mixed pairs from four different kinds of DNA also will form four distinct types of final product when cooled back down. A heteroduplex is formed, which means that it is not the original DNA but formed by a mixture.

Without the matching property, the two cannot form a hydrogen bond and instead form a small kink. The kink provides enough physical difference that it can be separated. A small kink can become a large disturbance if the temperature is increased slightly. As the temperature is increased, the hydrogen bonds nearby are also dissociated, creating a broken zipper effect.

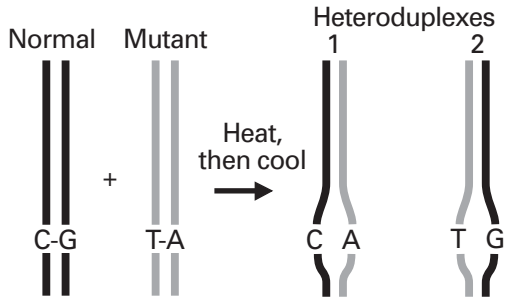


Figure 26. Single-point mutation (SNP) compares DNA by forming mismatches. DNA from two individuals with one base difference are heated and cooled to form heteroduplexes. If the DNAs were identical, heteroduplexes could not form.

Figure 27 illustrates how the capillary is placed on a heating plate; an increase in temperature unzips the zipper slightly to make the two fragments more different.

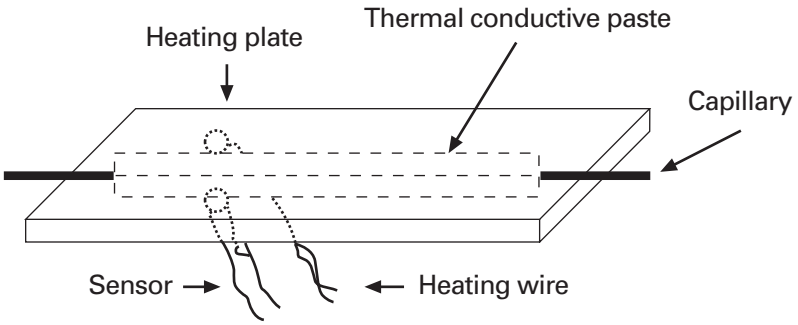
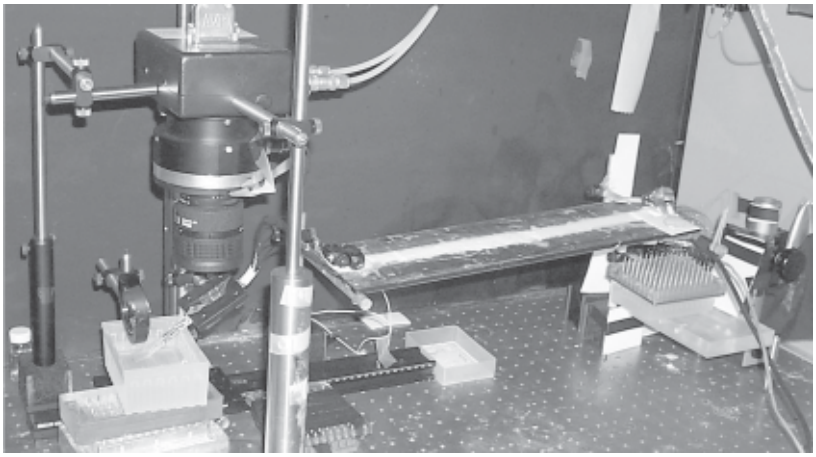


Figure 27. The capillary is placed on a heating plate in single-point mutation.



In single-point mutation, the 96 capillaries are on top of a heating plate to increase the temperature as the sample is separated.

Separations occur at different temperatures. For one mutation, 69° C is needed, for another the temperature is slightly below 68° C, and for another it is 65° C. The temperature needed is slightly different depending on the mutation (Figure 28).

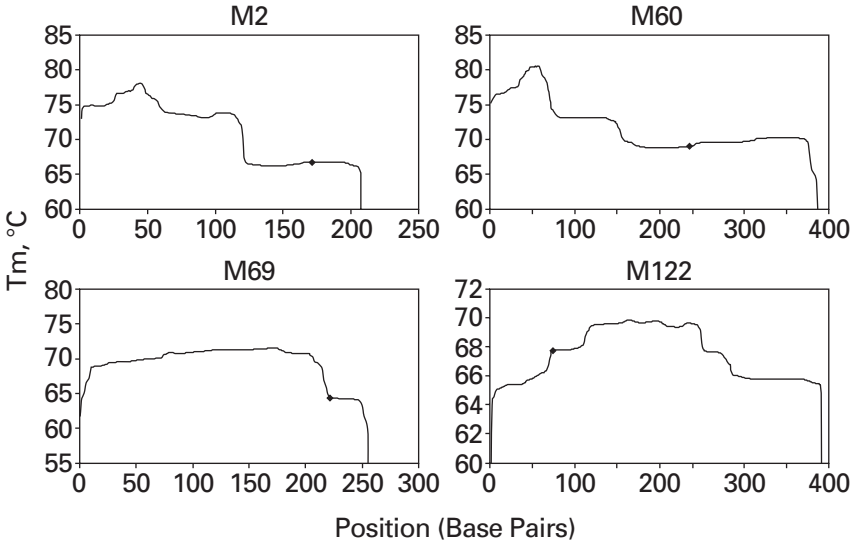


Figure 28. Mutations separate at different temperatures.

By changing the temperature throughout the run, as in gradient elution or temperature programming in gas chromatography, the samples at any one time arrive at the proper temperature for results.

Figure 29a shows two of the same DNA on the bottom and two different DNA samples on the top, so one peak versus four peaks. It is immediately known that the top sample has two DNA and the bottom only one.

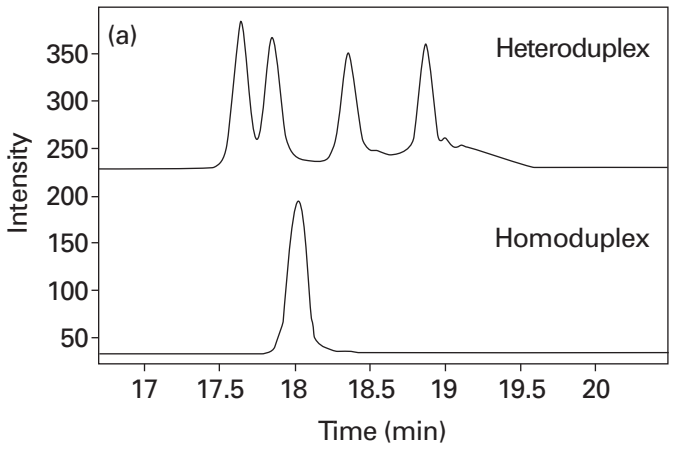


Figure 29a. A plot visually shows one DNA sample (bottom) and a sample with two DNA (top).

If conditions are not exactly right, four distinct peaks may not be seen (Figure 29b). A large glob is actually a combination of the four peaks. Even in this case, it is easy to recognize that the top sample contains more than one kind of DNA.

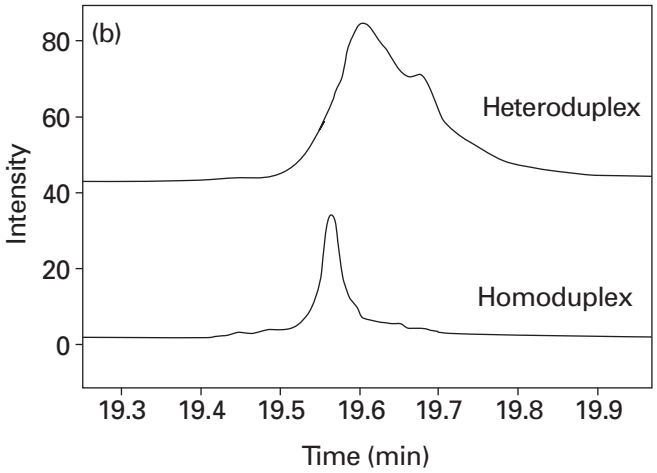


Figure 29b. A plot showing differences exist even when distinct peaks are not formed.

Without *identifying* DNA, this technique *matches* DNA; the plots show some separation and whether the samples are identical or not—even if only one of the bases is different. Figure 29c–e show various combinations of two DNAs, and in all cases, it is very easy to recognize, and juries can accept, even without computer analysis, that the two are different traces.

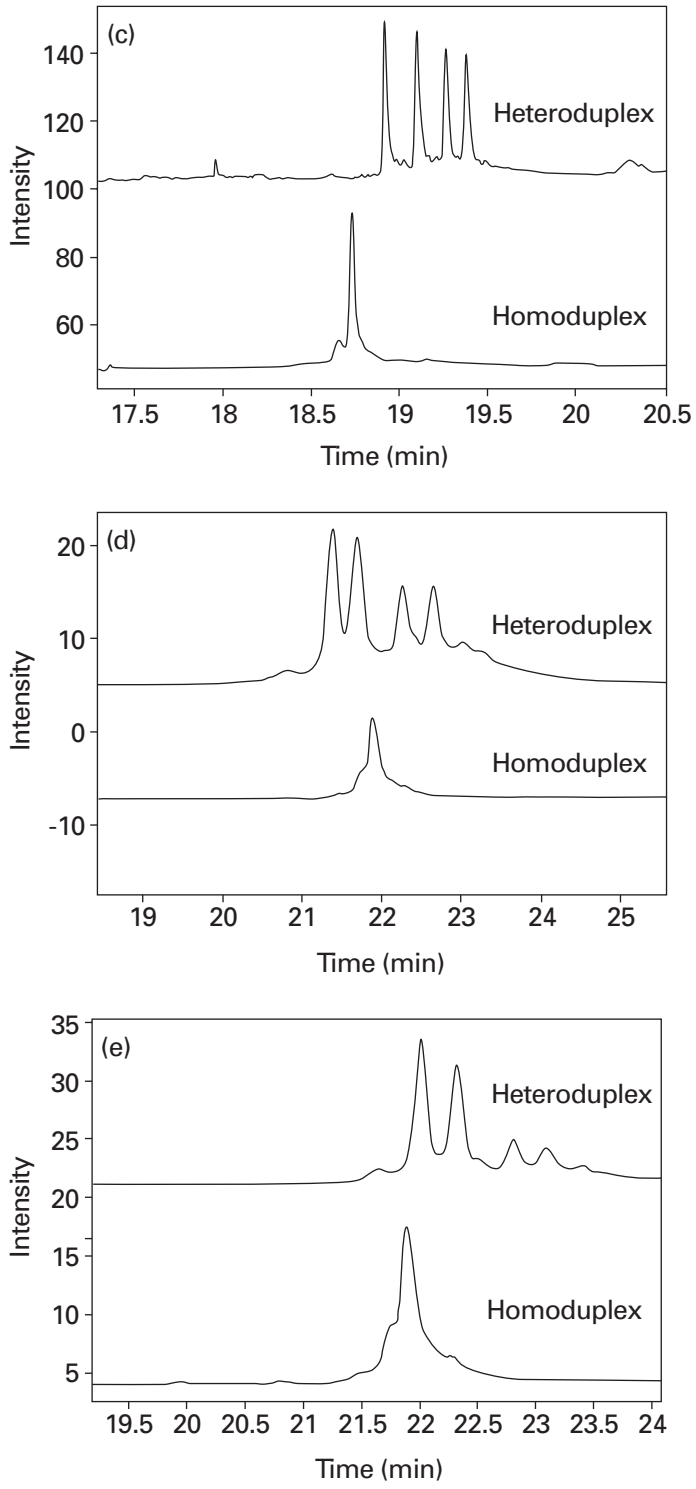


Figure 29c–e. A series of experiments show that the technique of matching DNA will produce plots with obvious differences when the samples differ.

Figure 30 shows four different genetic regions on the human genome. These repeat regions are not related to any genes, so they represent healthy individuals with different genetic coding in the chromosomes. The asterisks mark the traits with distinct differences from the standard. For each individual, there are slightly different locations, slightly different markers.

DNA fingerprinting requires about 15 regions. The experiment, for example, could be done in four different capillaries with four regions each. Using 96 capillaries, 24 samples could be genetically typed simultaneously within an hour to produce individual DNA fingerprints.

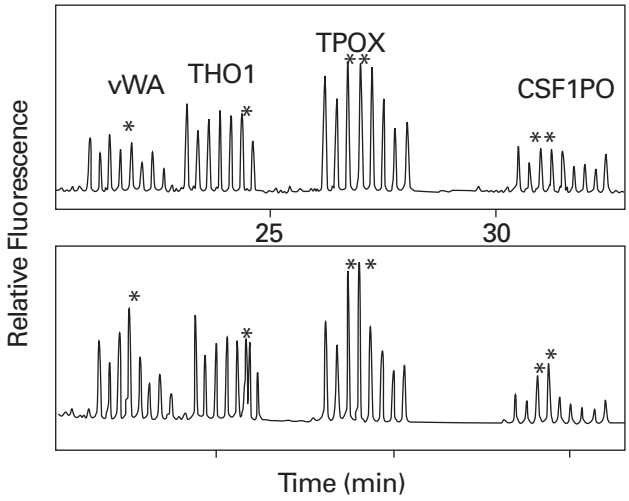


Figure 30. On-line PCR-CE for CTTv typing.* The upper electropherogram shows the on-line injection and separation of CTTv standard allelic ladder only. The lower one shows the result of on-line PCR, coinjection of standard allelic ladder and PCR products, and CE separation. Notice the change of relative peak intensities in the peaks marked with *. The genotype is named by the number of repeat sequence contained in the allele. The results are: vWA—16,16; THO1—9.3,9.3; TPOX—8,9; and CSF1PO—9,10.

With a DNA sequencing instrument, newer techniques of coinjecting a standard and the sample can be implemented and confirm with absolute confidence that a peak is a match or not. Two DNA samples by single-nucleotide polymorphism, so-called snip analysis, can be compared, identifying small genetic variations or mutations in an individual's DNA sequence, and single-mutation analysis can still be used to compare peak patterns.

*PCR-CE—polymerase chain reaction capillary electrophoresis
CTTv—the loci CSF1PO, TPOX, THO1, vWA

NOTES

Capillary Electrophoresis

3. Combinatorial Separation*

Learning Objectives

At the end of this section, the viewer should be able to:

- Recognize the importance of the Human Genome Project in driving the creation of high speed, high throughput DNA sequencing instrumentation and separating materials.
- Recognize the speeds that can be expected with 96-well capillary electrophoresis.
- Describe combinatorial separation.
- Recognize the areas in which high throughput combinatorial separations are used.
- Explain the problems with the 96-well capillary absorption detector and how these problems are solved.
- Describe the concentration process.
- Define peptide and explain how peptide fragments are made.
- Describe how peptide mapping takes advantage of the combinatorial separation conditions enabled through use of 96 capillary CE.
- Recognize how HPLC and multiplex CE can complement each other in giving a detailed history of cell extracts.
- Recognize that the testing of chemical content of cells is more interesting in terms of the history of the tissue and the individual, not just the genetic composition of the cell.

*Prepared by E. S. Yeung, W. Zhong, Y. Zhang, X. Gong, Y. He, S. Kang, K. Chan, H. Issaq, H. Zhang, and R. C. Larock, Ames Lab, Iowa State University, U.S. Department of Energy, National Institutes of Health

- Describe how the high speed, high throughput combinatorial separation of CE makes it practical to run this kind of comprehensive test, when it was not practical in the past.

- Explain the process of taking fractions and running a preconcentration on the fractions to obtain the desired separations.

- Describe chiral molecules and explain why is it important to separate them.

- Describe both an expensive and an inexpensive way to separate chirals.

- Describe how an additive like cyclodextrin separates chirals.

- Explain the optimization of the chiral separation solution through combinatorial CE.

- State the goal of synthesis.

- Describe the synthesis process that has been done for years versus the combinatorial synthesis process using high speed, high throughput.

- List the advantages of combinatorial synthesis.

- Explain nonaqueous electrophoresis and list its advantages.

- Describe ways in which high speed, high throughput with combinatorial synthesis in nonaqueous CE enables researchers.

NOTES

High Speed, High Throughput Combinatorial Separations

- Initially driven by the Human Genome Project for DNA sequencing or genotyping, but also . . .
- Clinical diagnosis
- Industrial processing
- Environmental monitoring
- Combinatorial synthesis
- Proteome, metabolome
- Methods development

In capillary electrophoresis, separations have been performed in a controlled laboratory setting down to the 100 μs level: 100 microseconds, two components, two peaks. The 96- or 384-capillary instrument enables high throughput. Although initially developed for the Human Genome Project, there are other areas where high speed and high throughput are needed for chemical separation:

- in clinical diagnosis, to ease the substantial throughput problem that exists with the number of tests run (e.g., on a single blood sample) and the number of patients (e.g., normal annual blood tests).

- in industry, production processes need to be monitored in terms of chemical composition and how things change throughout the process: catalysts may change, temperature may change, feedstock may change. Sampling the chemical composition at each step in the order of minutes or seconds to maintain the optimum can save millions of dollars.

- in environmental monitoring, air, water, and soil require many samples and many different types of tests.

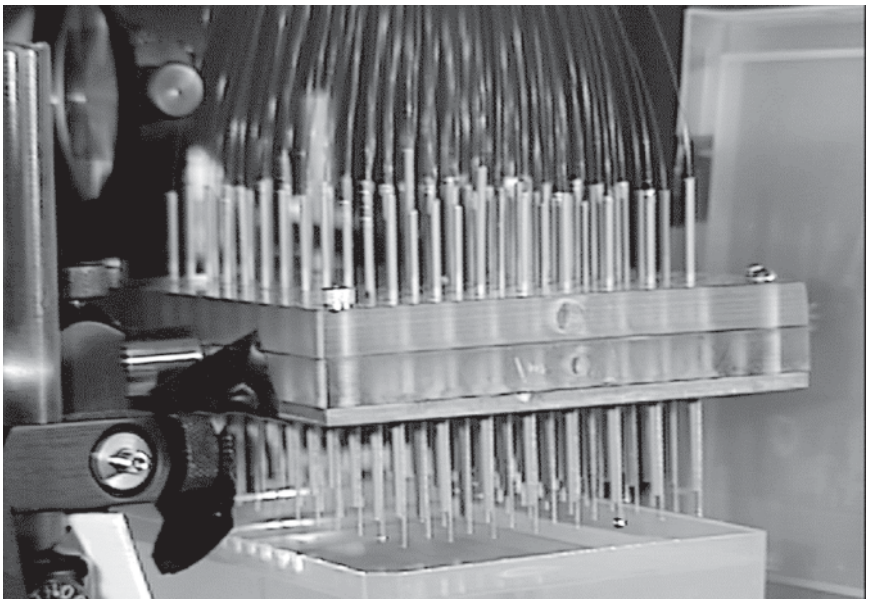
- in combinatorial synthesis, thousands and thousands of possible compounds are produced for potential future drugs; each has to be analyzed in terms of composition and purity.

- in proteome and metabolome analysis. The genome is just a template of all the parts within our genetic system, but from that parts list (e.g., similar to assembling an automobile), the proteome or proteins do all the assembly work. The same genetic code (DNA) does not necessarily produce the same proteins. The proteins in the eyes, ears, hair, and skin cells are different and need to be analyzed. There are forensic reasons for analyzing proteins instead of just DNAs.

  Metabolome is the result of the function of the protein (metabolites/metabolism). Even with the same person, with the same proteins, the function may differ. With DNA as the basis, proteome and metabolome analysis involves the next order of complexity and many more orders of magnitude in samples and speed requirements.

- in methods development. Combinatorial separation is a concept that can allow development of separation methods. One of the most time consuming and difficult tasks in chemical analysis, GC and LC included, is to find the right condition to separate the two compounds of interest.

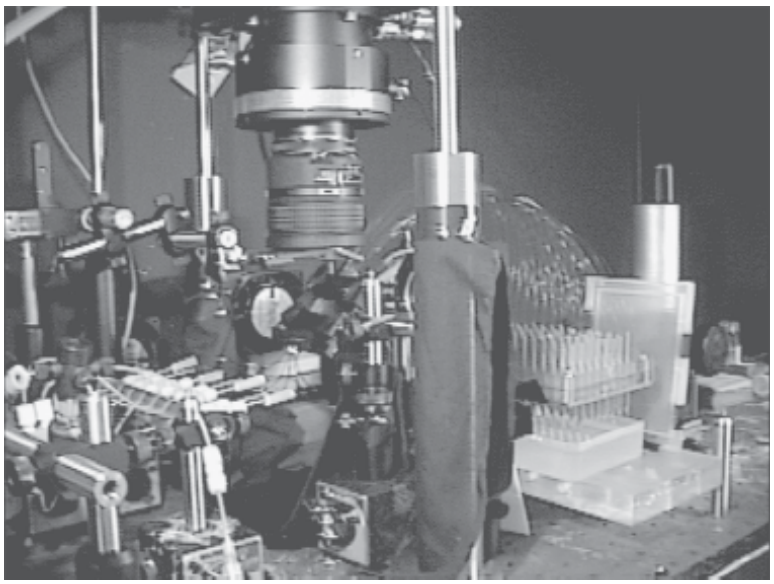
  It can take weeks to try different solvents, composition, and columns to get the right separation. With 96 capillaries, one can experiment with 96 different separational additions at one time. In a single run, one can arrive at the optimum separation condition—the reason for combinatorial separations.



The 96-sample injection system can do 96 of the same experiment or 96 different experiments simultaneously.

NOTES

DNA sequencing is based on fluorescence. A laser excites DNA molecules that are labeled or tagged with dye molecules and then emit light, so they can be seen. Fluorescence is very powerful but only works with DNA molecules; for other molecules, it is difficult to incorporate a fluorescent label, and if they do not fluoresce, they cannot be seen by a camcorder.

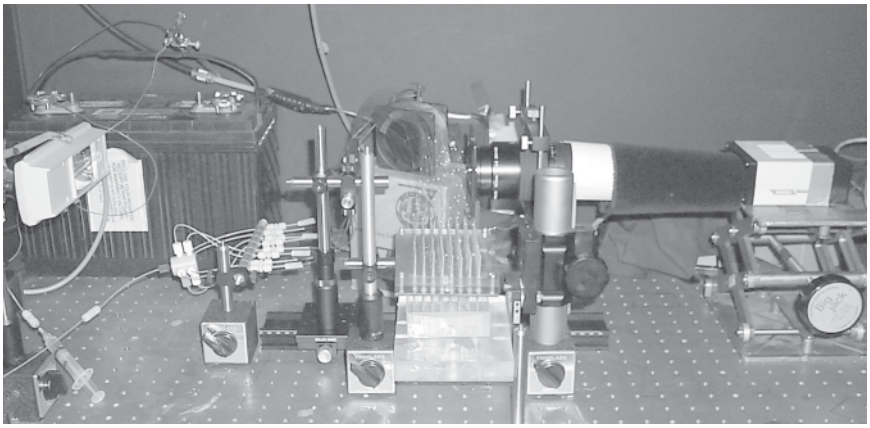


Ninety-six capillary array electrophoresis.

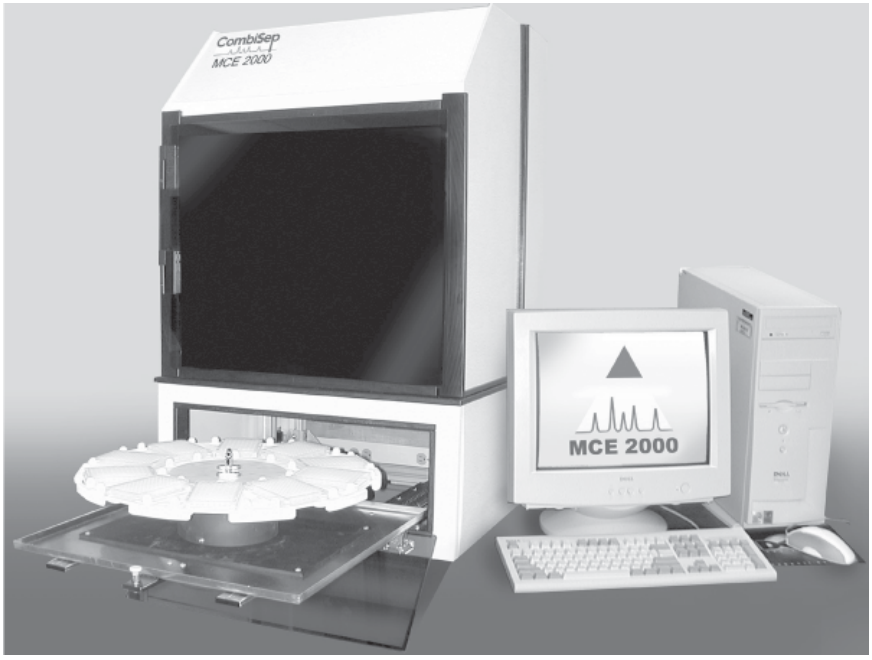
Because 90% of the compounds that absorb light do not fluoresce, a new type of instrument was developed six to eight years ago based on absorption detection.

With the same 96-capillary setup and the polyimide coating burned off, a light source—usually a mercury or tungsten lamp—replaces the laser, transmits light through the capillaries, through a camera lens, to the camcorder. A different kind of device, it measures the light absorption of any compounds that pass through the system; instead of viewing 10% of all possible compounds, 95–98% of them can be seen.

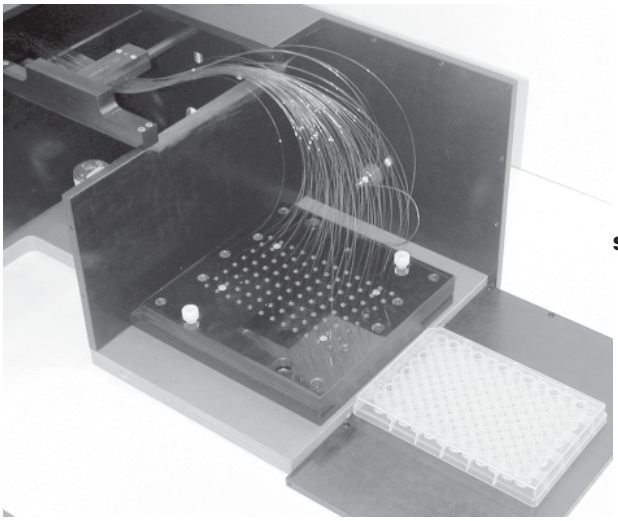
Within the last three years, a capillary absorption detector was developed—one instrument with 96 capillaries inside. It resembles a DNA sequencing instrument but measures absorption, not fluorescence. The capillary absorption detector takes the place of 96 HPLC instruments because entire trays are analyzed at one time. HPLC would require 96 setups, bigger pumps, and more solvents.



The 96-capillary absorption detector was developed six to eight years ago.



The 96-capillary absorption detector (= 96 CE or 96 HPLC) was developed about three years ago (www.CombiSep.com).



Inside are 96 independent CE channels. The computer controls movement of the trays while chemistry on the samples and feeding the trays are done by technicians or robotics.

Detector Performance
(as good as any single CE instrument)

▪ Crosstalk	▪ Visible LOD
0.2%	0.000013 AU
	18 nM R6G

The capillary absorption detector has some performance challenges, and one of these is interference among capillaries or crosstalk. Because there are 96 capillaries, it is critical that the light one sees is related to a specific capillary, not just a smear of image. Precise focusing between the camera and the camcorder is essential.

The capillaries are tiny—50 μm in internal diameter—so the focus is on something extremely fine over a large area. With optical innovation, crosstalk can be reduced down to about 0.2%, which is negligible because most measurements require about 1% uncertainty.

Fluorescence has good detection limits, but absorption is satisfactory—18 nM $\times$ 10^{-9} molar concentration of better absorbing material. The detection is as good as any capillary instrument.

There is still a problem: absorption is 18 nM $\times$ 10^{-9} but fluorescence is 18 nM $\times$ 10^{-12}— a 3 orders of magnitude difference in sensitivity. (Although fluorescence only works if the molecules fluoresce or the detector would not work at all.)

Detector Performance
(better than fluorescence derivatization)
Anal. Chem. **2000,** *72,* 1023–1030

Approaching a Million-Fold Sensitivity Increase in Capillary Electrophoresis with Direct Ultraviolet Detection: Cation-Selective Exhaustive Injection and Sweeping

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Published research shows that samples can be concentrated up to a million times before analysis. That means if your sample is 18 fM $\times$ 10^{-15}, it will become 18 nM and will be seen by the detector.

So while optically no adjustment can be made, these special techniques, unique to capillary electrophoresis, improve detection to unimaginable regimes for any kind of measurement up to a million times of concentration.

Example: A sample vial might be 10 µl in volume—typical pipetting—but only 10 nl of material will be injected, using only 1 part of 1,000 parts of total available sample. That is not very efficient. These techniques allow injecting the entire 10 µl sample into the capillary, giving 1,000 times the original material.

Injecting just the sample will not give a single narrow band; it will be a very long band and appear as a smear in the chromatogram. Short injection plugs are needed in order to retain sharp peaks.

After injecting into the capillary, the zone can be compacted by applying the electric field and using the right buffer. The zone can be squeezed into a tiny narrow plug.

Even though the tubes are tiny, small amounts of material can be detected or not by concentrating the material. The concentration process is a sophisticated technique that can be implemented in almost any capillary electrophoresis instrument. Computer programs switch buffers, change the electric field, and perform other operations totally transparent to the user. Published research will easily adapt to commercial instruments when software programs are developed.

Another concern unique to capillary electrophoresis is that the same peak can come out at different times for repeated runs or from one capillary to the next capillary because of electroosmotic flow. The electroosmotic flow—the moving sheath or skin—can change in speed from one experiment to another and particularly from one capillary to another because the chemical history of each capillary is slightly different.

The molecule has a reproducible charge and size, so that part of the movement is reproducible, but the capillary has a different charge at different times and different temperatures. The capillary wall is constantly changing during the separation process, as the capillary is used and while the sample is being injected.

In an experiment involving 96 capillaries, each data point is the migration time or how long it takes the capillary to reach the detector (Figure 31). One component is marked with squares and the other is marked with triangles. The solid or outlined squares and triangles indicate two separate runs.

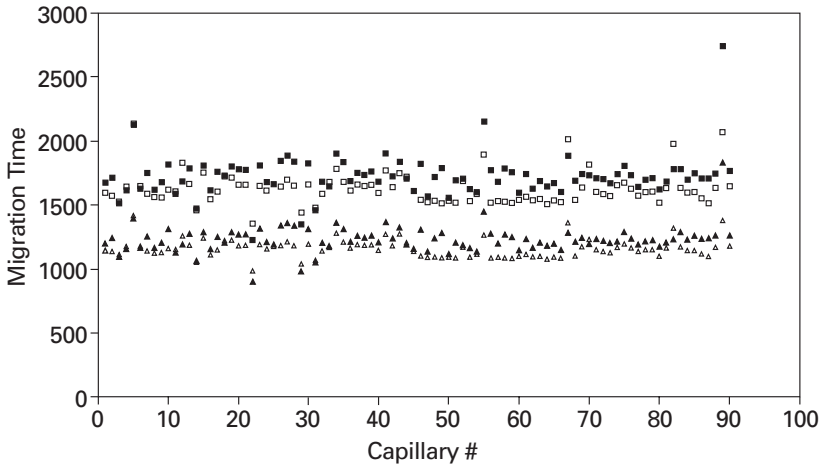


Figure 31. Raw migration times.

Note that within one run and with different capillaries, solid squares are varying all over, and from run to run, even the same capillary does not have reproducible time. The capillary surface is difficult to control; the flow rate is not constant, affecting the ability to determine times and identify compounds.

In GC and LC, results are reproducible because the flow rate is constant with a gas cylinder or pump. The trick in CE is to measure the velocity of the electroosmotic flow, make the correction, and realign the raw data for normalized migration times (Figure 32).

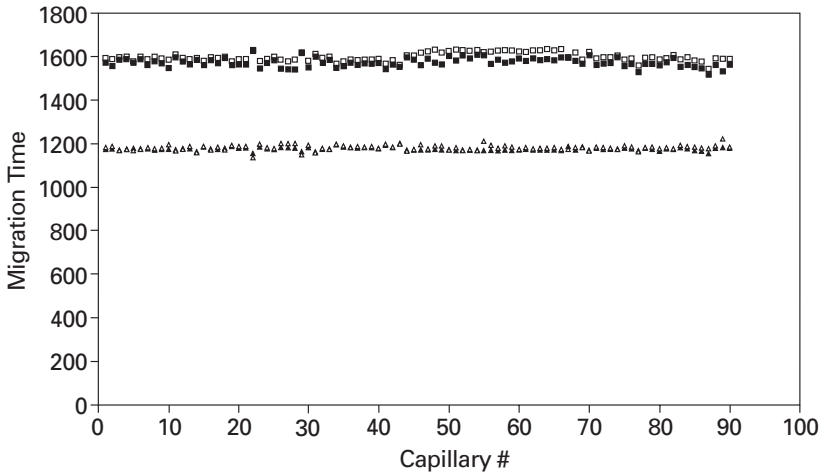


Figure 32. Normalized migration times.

When the computer calculates the electroosmotic flow and makes the appropriate correction, data align to within 1%, and from run to run or from capillary to capillary, results are reproducible.

Comprehensive Peptide Mapping in Capillary Electrophoresis

Figure 33 is an example of peptide mapping or the analysis of proteins. Proteins are large, complicated molecules with 20 or so alphabets, all with different shapes, compared to the four alphabets for DNA sequencing.

A reliable way to identify protein is to look at the individual segments or peptides of the protein. One protein may have 10 peptide fragments and another may have 1,000, depending on how the protein is chopped up. If chopped in a known manner, the protein can be reassembled like a jigsaw puzzle by computer in a process called bioinformatics.

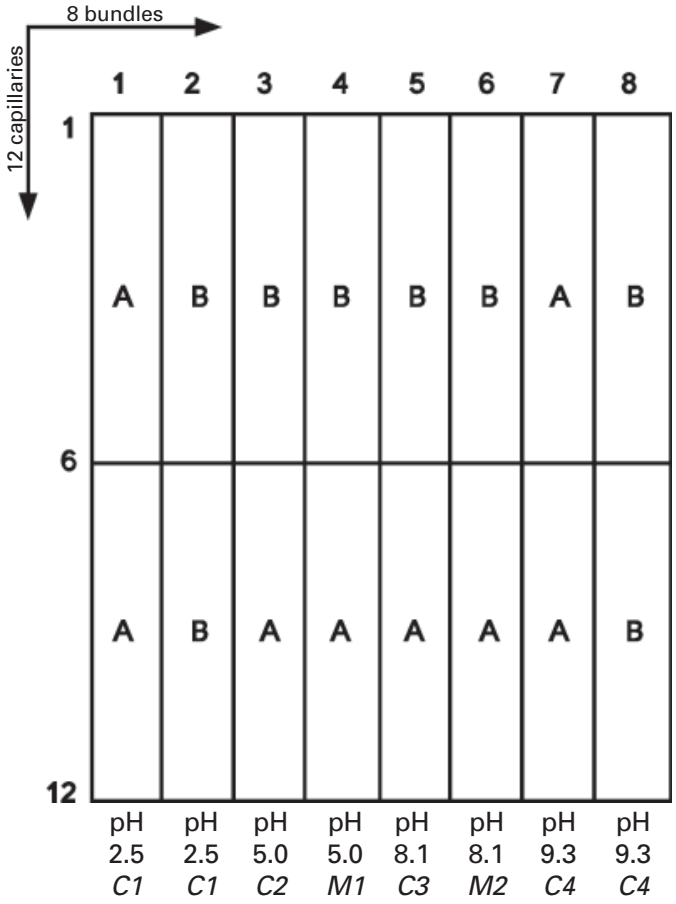


Figure 33. Comprehensive peptide mapping (combinatorial separation conditions).

Peptide fragments are created by enzymes that cut proteins into small fragments. This is a natural process; for example, proteins in the stomach are cut into digestible pieces by enzymes, such as pepsin and trypsin, so protein can be readily absorbed into the body through the intestines.

Peptides are not like DNA sequencing fragments that are so regular and reproducible. Each part is basically several amino acids with acid and base qualities.

Acids and bases are separated by changing the pH. As long as the pH is altered so one is more positive and one is more negative, they will separate, but separating 100 to 1,000 peptides in a typical protein digest—several hundred pieces of jigsaw puzzle—would require many changes in pH.

With 96 capillaries, one can preset a combination of separation conditions (combinatorial separation conditions). One of the conditions that needs to change is pH, so in this example, four different pH values were chosen as buffers: 2.5, 5.0, 8.1, and 9.3 (Figure 33). It is not necessary to use other pH values (e.g., 5.1, 5.2, 5.3) because titration curves usually span about two pH units.

Some of the peptides are neutral (neither acid nor base), so pH cannot be used to adjust them. Neutral peptides will separate by micellar electrokinetic chromatography. Adding some detergents to the pH 5.0 and 8.1 buffers creates a separation possibility for neutral species as well.

Figure 33 shows the six predetermined separation conditions that were selected (the first and eighth are repeats to fit the standard microtiter plate format [$8 \times 12 = 96$]). The conditions were selected to impose differences in the peptides so that they will have a chance of separating without trying all the separation possibilities.

Figure 34 is an example of a peptide map for lactoglobulin, a common protein with variants A, B, and C. The 20 or so alphabets refer to different amino acids that are linked together in proteins. Each of the variants differs slightly by an amino acid substituted here or there to make each protein different from the other.

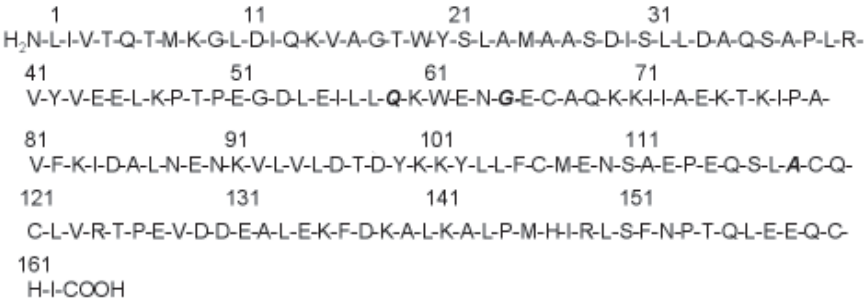


Figure 34. Comprehensive peptide mapping (fingerprint for expression, mutation).
Amino acid sequence of bovine β-lactoglobulin B.
Variants A and B (BLGA and BLGB) differ at two sites: Asp 64 in A is changed to Gly in B, and Val 118 in A is changed to Ala in B. Variants B and C (BLGB and BLGC) differ at one site: Gln 59 in B is changed to His in C.

Normally the differences would not be recognized, but if the protein is cut into small pieces, then one region would differ from the other. Rather than looking for one amino acid out of a hundred, it is easier to recognize one amino acid out of a few—seven or eight.

With the six predetermined conditions, there will be six different electropherograms because some of the peptides are positive, some negative, some neutral (Figure 35).

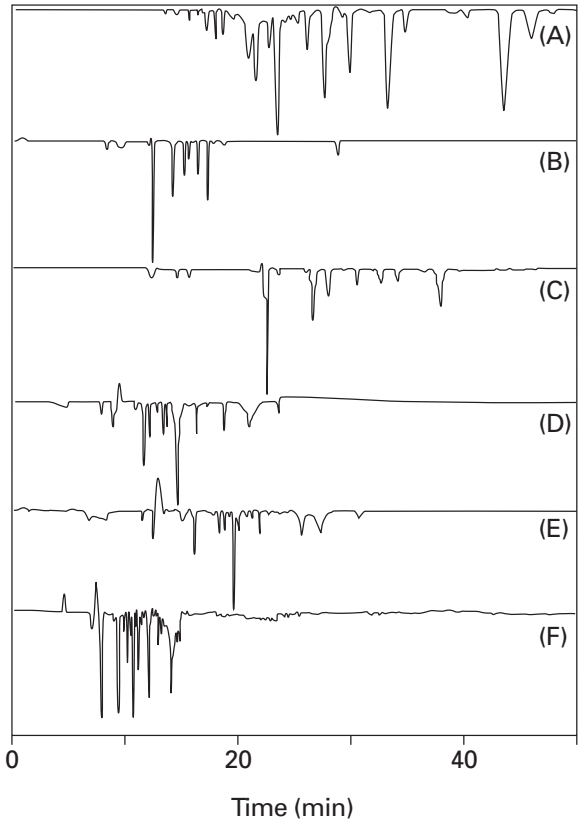


Figure 35. Comprehensive peptide mapping (6 complementary CE conditions).

Similar to genotyping or DNA fingerprinting, the six different patterns should tell which protein the original sample contains.

The power of protein fingerprinting is shown in Figure 36. The pH 8.1 condition gives somewhat similar peptide maps for lactoglobulin A and B; the similarities are expected because they have almost identical amino acids. On close examination, there are very distinct differences in peaks between the two electropherograms.

The other five conditions also show distinct differences, so with an extremely high level of confidence, one can identify proteins based on capillary electrophoresis.

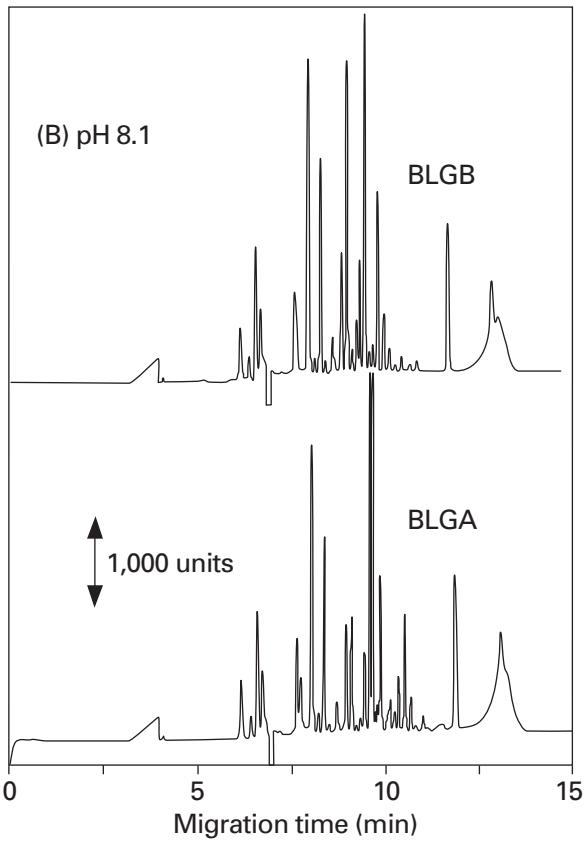


Figure 36. Comprehensive peptide mapping (detecting minor changes in proteins).

Using Complementary HPLC and Multiplex CE for a Detailed History of Cell Extracts

NOTES

A valuable tool in forensics is extracts from cells because it may be important to know the composition of human or animal tissues. Samples will show completely different cellular contents before and after an individual has ingested certain foods; samples will differ in an individual with high blood pressure during waking and sleeping periods; samples will show different sugar contents in diabetics; and so forth. Testing the actual chemical content of cells reveals genetic composition and the history of an individual.

Basically, all the chemical and biochemical species can be extracted, including proteins and peptides and other smaller molecules in cells—a very complicated process with thousands of components.

HPLC/Multiplex CE/UV for Comprehensive Cellular Mapping

- HPLC—first dimension
  - Separation based on hydrophobicity
  - High separation efficiency and on-line detection
- Multiplex CE—second dimension
  - Separation of HPLC fractions based on the charge-to-mass ratio
  - High separation efficiency and on-line detection
  - High throughput by capillary array

HPLC provides one mode of separation but mostly for nonpolar compounds. A second dimension or separation based on capillary electrophoresis gives complementary effects based on size and charge. Something that separates in HPLC may not separate in CE and vice versa, but there is a good chance all components can be resolved with a combination of the two.

An HPLC chromatogram of cancer cell extracts shows good resolution and many tens of peaks (Figure 37). Detection was by UV absorption at 214 nm where almost all organic functional groups will absorb light and almost everything that is present can be seen. The chromatogram shows good separation but indicates many components are overlapping and unresolved as individual peaks.

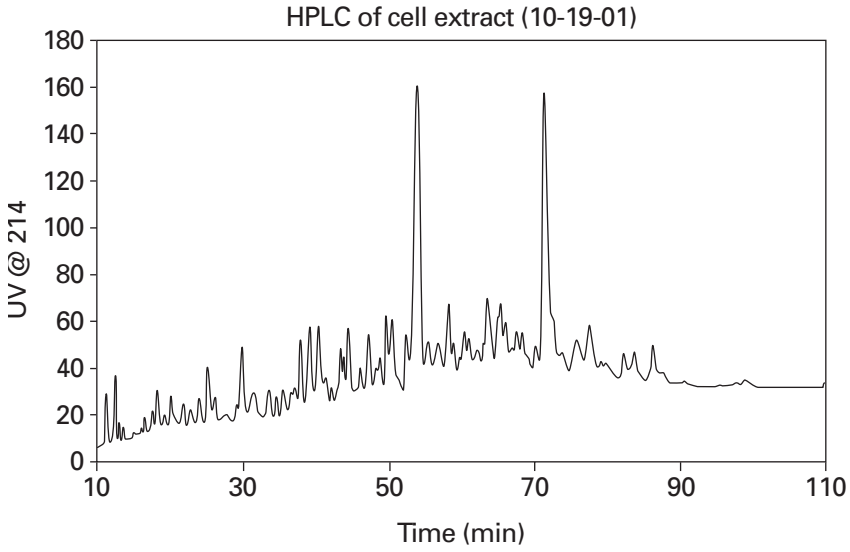


Figure 37. Separation of cancer cell extracts by HPLC.

Collecting fractions means that a vial placed at the end of the HPLC column collects the sample for every minute. The vials contain all the unresolved components under each minute segment and are then further analyzed by capillary electrophoresis.

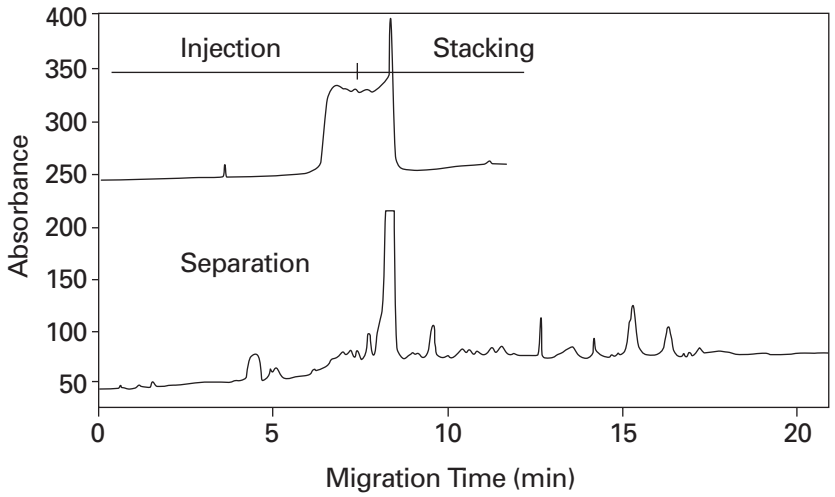
Initially the process was extremely tedious and took a long time: there was a liquid chromatography run, an hour and a half, and then separate CE runs, half an hour to an hour for each vial.

With the technological capability of running 96 samples at once, each vial of collected fractions simply goes into the tray and all the samples are analyzed at the same time in the same instrument. The time savings is significant: an hour and a half for the HPLC run, and only an additional hour for the final results.

After the components come out at the end of the HPLC column, they are tremendously diluted by the solvent. Therefore, capillary electrophoresis may not have sufficient sensitivity to see peaks even if they are resolved.

A preconcentration technique is programmed into the computer. The fraction is collected, injected in the capillary tube, and then squeezed into narrow bands before separating.

Peaks would not seen in normal capillary electrophoresis, but after the preconcentration process, the sensitivity is enhanced 100 to 1,000 times (Figure 38).



Conditions: capillary (35 + 20) cm × 75 μm i.d.; injection, 20 cm × 7 min stacking voltage: –10 kV; separation voltage: +10 kV.

Figure 38. Large volume sample stacking and separation of HPLC fractions.

The electropherograms are slightly different from each capillary because each sample was taken at a different time during the HPLC run and contains different sets of molecules (Figure 39).

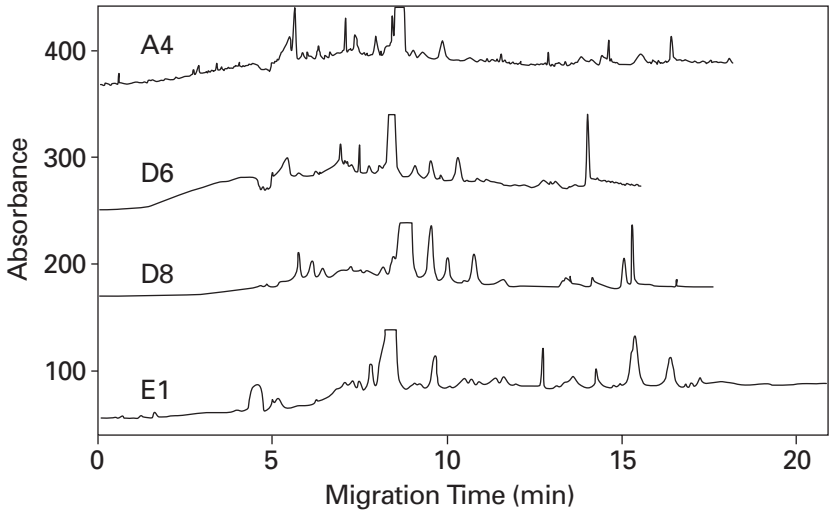
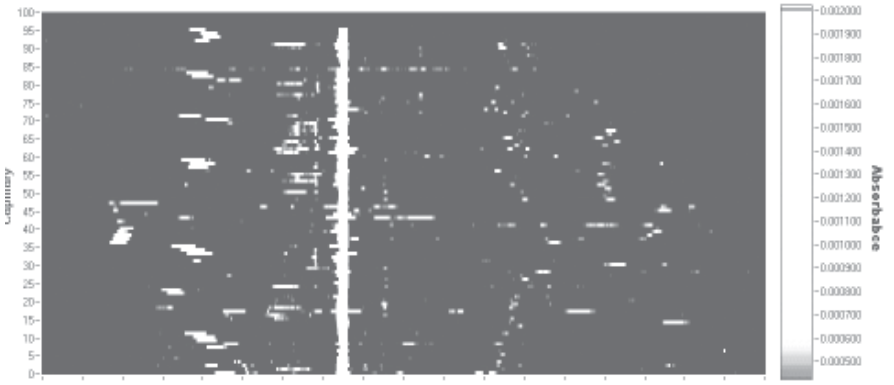


Figure 39. LVSS-CZE of four HPLC fractions of cancer cell extracts.

The A, B, C, D coding is the row number and the number is the column number used to identify each of the 96 wells. All the data is stored in the computer.

It is difficult to visualize 96 wells as separate electropherograms, but replotting them as an intensity scale on the computer helps perceive the whole picture at the same time.



Computer plot derived from LVSS-CZE of four HPLC fractions of cancer cell extracts.

The photo shows a computer replot of the 96 capillaries from 96 different times during HPLC. Both HPLC time and CE time are indicated; the power of collecting fractions can be seen by the very resolved peaks, which are sometimes resolved by liquid chromatography and at other times resolved by capillary electrophoresis.

If 100 peaks are resolved by capillary electrophoresis and 100 by liquid chromatography, two dimensions mean 10,000 peaks can be resolved (100 × 100).

From cell tissue extracts, much is learned about the metabolism and health of an individual and by looking at cells, links can be found to certain kinds of cancer. This comprehensive cellular mapping would be possible with a single capillary electrophoresis instrument, but it would not be practical because of the time involved.

Chiral Separations by Capillary Electrophoresis

NOTES

It takes experimentation to increase the opportunity of separating compounds and to arrive at the best condition for separation (e.g., using six conditions to produce six different kinds of peptide maps).

Chiral separations, separating left-handed and right-handed or asymmetrical molecules, are the most difficult because, by definition, they have the same chemical composition—size, charge, everything is similar. Like left and right hands, they are not superimposable on each other, and it takes sophisticated methods to separate them.

Liquid chromatography uses specialized columns, so left-handed and right-handed gloves were placed on the column and as the analytes passed through the column, molecules separated by going to the corresponding glove.

The columns are sophisticated and expensive ($2,000 to $3,000 each); one column can separate only one type of compound, but there are thousands of different kinds of compounds. Large pharmaceutical companies can afford to have a collection of 100 chiral columns at $3,000 each, but such an investment is unusual.

Identifying left- and right-handed molecules will give the most detailed level of discrimination and has forensic relevance.

Prescription drugs and illicit drugs are generally pure with molecules that are one-handed or the other. However, after the drugs are ingested and go through normal metabolism in the body, right-handed forms can be produced from the left-handed original form and vice versa.

Metabolism of the drugs is reflected in the left- or right-handed ratio of products that are formed, and that tells, for example, how long ago the drugs were taken, what else in the body caused metabolism to be functional or not, and whether there was any doping or not.

Once metabolized, left- or right-handed drugs will actually go to a certain steady state in the body. For example, an Olympic athlete, tested for using performance-enhancing drugs, may claim these are

natural components in the body. Because of metabolism, a drug will not show the same ratio of left- and right-handed molecules, so it is easy to tell the drug was ingested recently and the body has not reached equilibrium or a steady state among these isomers.

Chiral separations are very powerful and used to follow the progress of drugs in particular. A well-known example is thalidomide, a very effective painkiller approved for use in Europe in the 1960s. Unfortunately, babies were born with severe birth defects when mothers took thalidomide during pregnancy. Thalidomide is a pure and very useful drug, except that manufacturers did not sort out the left-handed and right-handed forms. Thalidomide is still used today and is an example of the importance of quality control in either producing pharmaceuticals or following the effects of pharmaceuticals in the body. To ensure drug safety, manufacturers are willing to spend millions of dollars to identify the proper form of the drug and test for contaminants.

Chiral separations can be done inexpensively with capillary zone electrophoresis by introducing an additive, cyclodextrin—a sugar complex or polysugar (Figure 40).

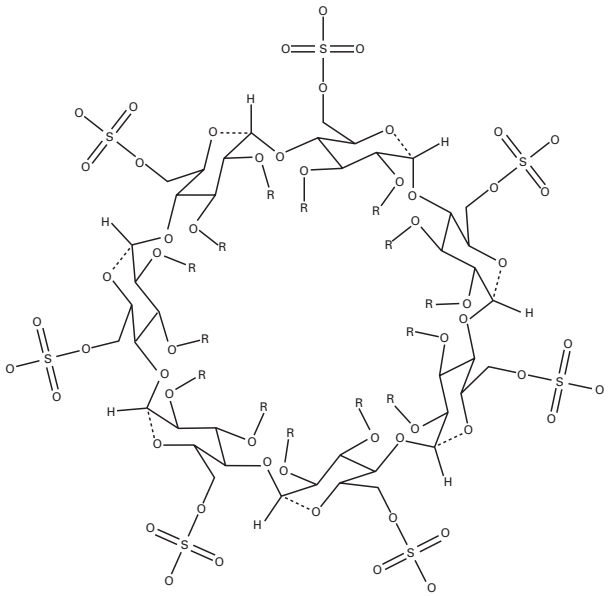


Figure 40. Chiral separations. Native and derivatives of α, β, γ cyclodextrins are known to be effective chiral selectors in CZE.

There are many sugar groups that give distinct glove property for the left and right hand because sugars are chiral, left-handed or right-handed. By having the left- or right-handed sugar, the molecule can be fit either properly or improperly and will separate.

The sugar ring has to be the right size to fit the molecule, even though there is a glove (e.g., any hand will fit in a large glove but not in a small glove). The different sizes of cyclodextrin are α, β, and γ, and different concentrations of this molecule are needed because some bind more or less strongly. The adjustable parameters include the various types of cyclodextrins and concentrations, so there are many trials.

Charged Resolving Agent Migration Model

Theoretical model for predicting mobility and separation selectivity as a function of the charged-CD concentration and pH

- For neutral and permanently charged analytes, pH would not affect resolution much.
- For weak acids and bases, either high or low pH would provide good resolution.
- There exists an optimum charged-CD concentration for maximum selectivity.

With 96 capillaries, all the trials can be done at the same time. Figures 41A–E show chemical compounds of 54 nonprescription drugs that can be purchased from the *SIGMA* catalog. The drugs have different chemical structures, but they're all chiral—left-handed or right-handed. Not intended for actual use as drugs, they are made in the cheapest way possible and are mixtures of left-handed and right-handed molecules.

To demonstrate how chiral separations are done inexpensively with capillary zone electrophoresis, five different cyclodextrin conditions were used to separate the drugs (Figures 42A–E). All five capillaries will not show good separation, but one of the conditions will give satisfactory results. By the rate of the peak areas, one can determine the rate of concentrations without trying all the 100 columns used for HPLC.

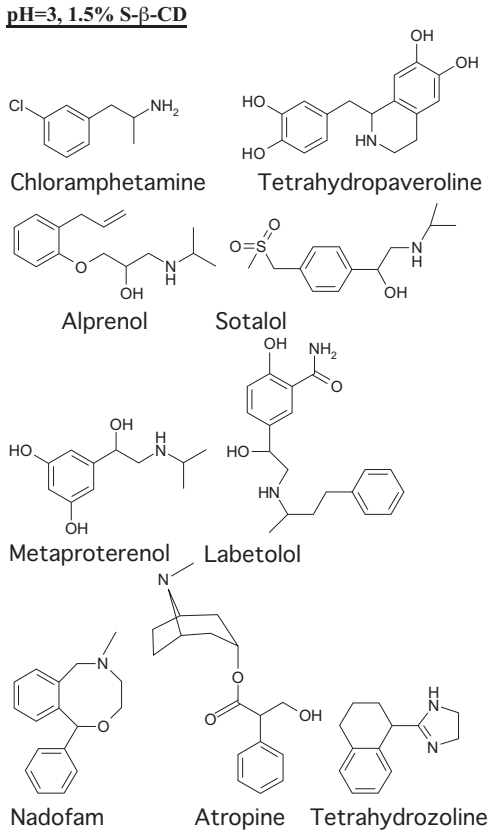


Figure 41A. Combinatorial chiral separation.

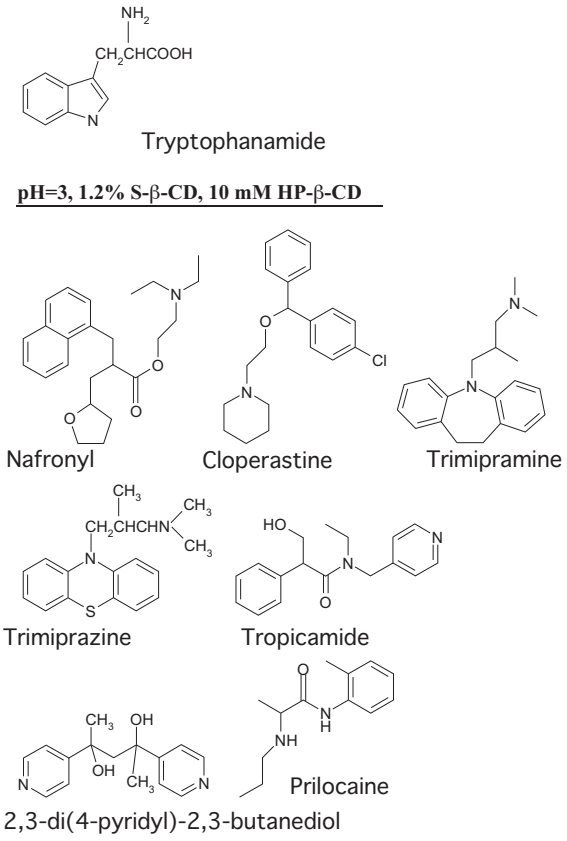


Figure 41B. Combinatorial chiral separation.

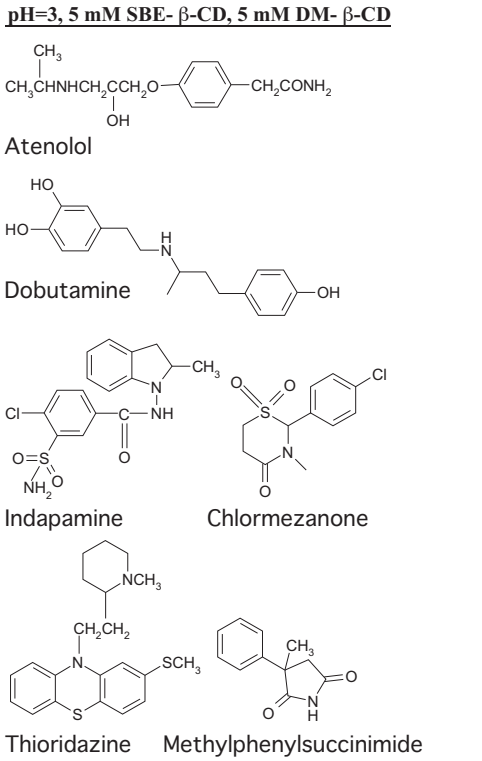


Figure 41C. Combinatorial chiral separation.

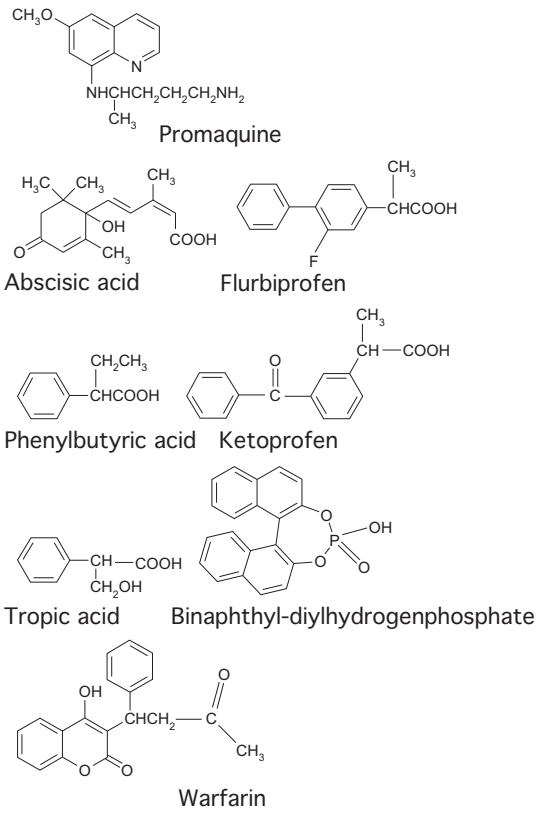


Figure 41D. Combinatorial chiral separation.

pH=3, 5 mM SBE- β-CD, 20 mM TM- β-CD

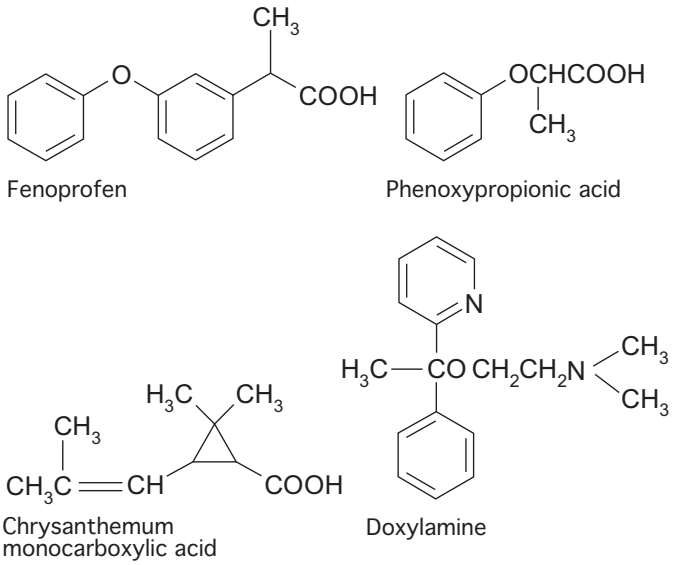


Figure 41D. Combinatorial chiral separation.

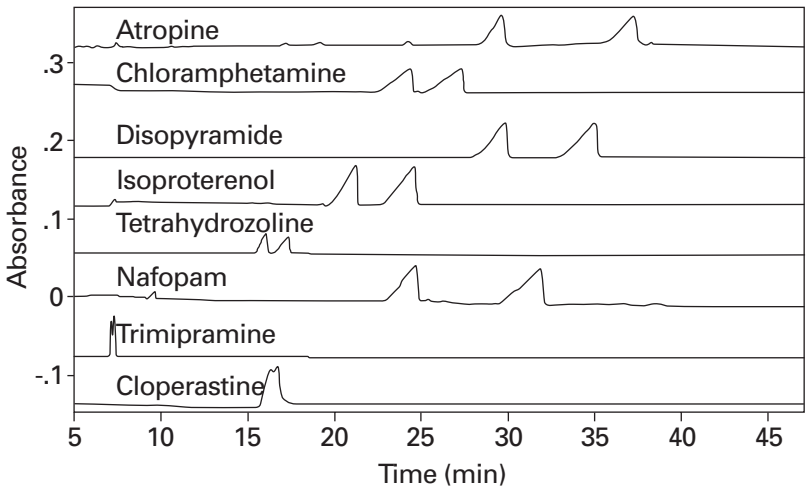


Figure 42A. Combinatorial chiral separation.

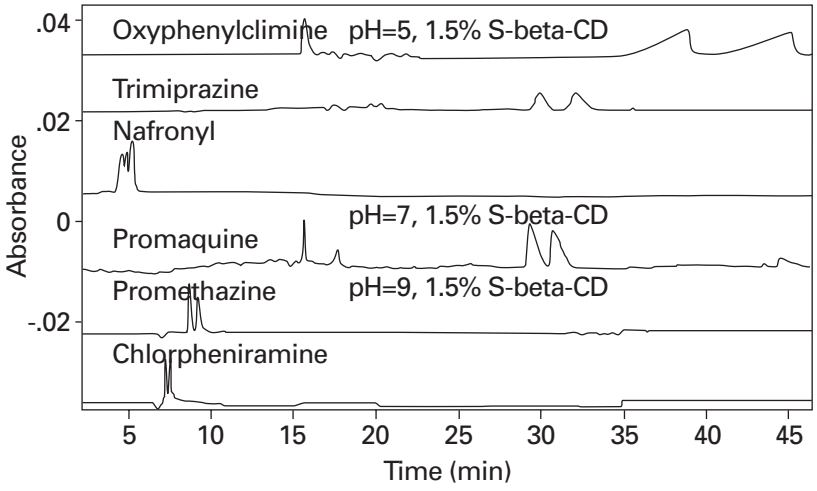


Figure 42B. Combinatorial chiral separation.

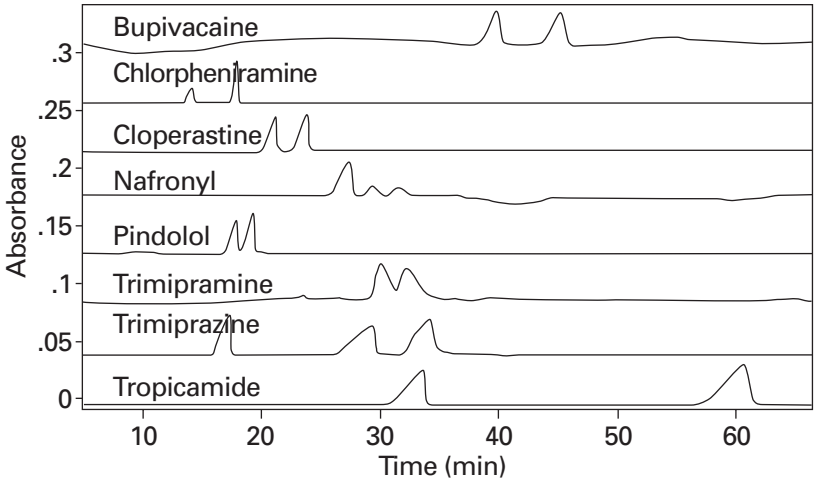


Figure 42C. Combinatorial chiral separation.

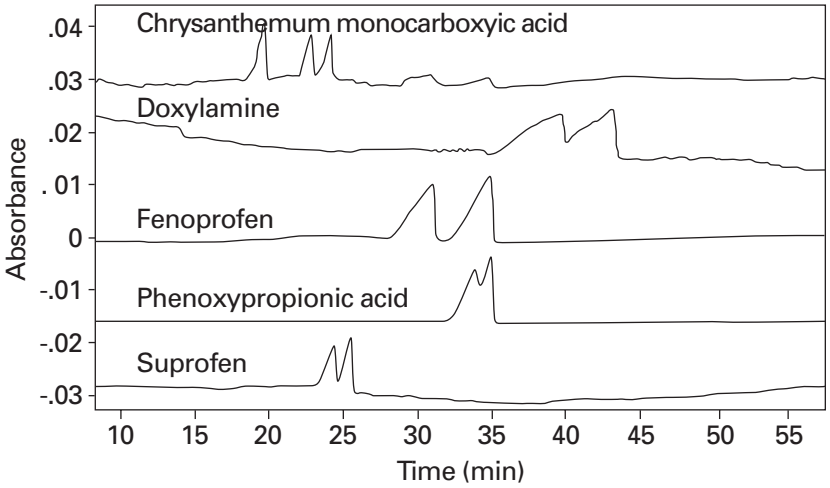


Figure 42D. Combinatorial chiral separation.

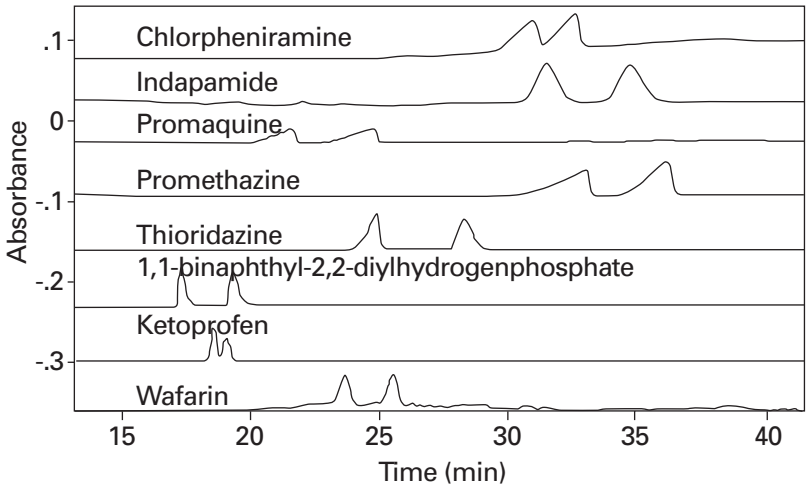


Figure 42E. Combinatorial chiral separation.

Combinatorial Synthesis by Capillary Electrophoresis

The goal in synthesis is to achieve the highest possible yield; so, organic chemists optimize the conditions of reactions to get the most product. Figure 43 illustrates such a reaction—a ring closure experiment—to produce a more useful chemical, a precursor to a drug.

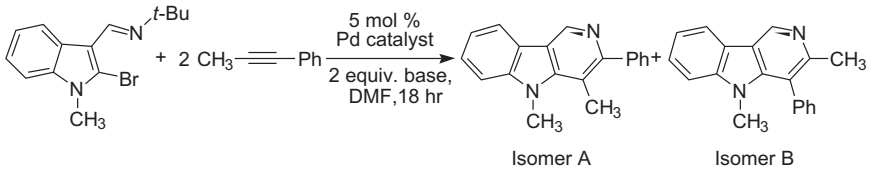


Figure 43. Combinatorial synthesis (nonaqueous CE for direct sampling from reactor).

The reaction is straightforward: there is an organic compound (left), and the common bond used to close the ring. Different catalysts were used, involving many palladium compounds and natural compounds that speed up the reaction. Different organic solvents were tried because sometimes polar solvents are better than nonpolar solvents to produce the most product.

The experiment was an actual organic chemistry project at Iowa State University. A graduate student took the two starting materials, added a predetermined solvent and a certain amount and type of catalyst, and ran the reaction overnight. The reaction took approximately 18 hours. After analyzing the mixture to determine whether the yield was satisfactory or not, the conditions were changed and the process repeated—another 18 hours. It takes months for one person in one lab to optimize a particular synthesis.

Such experiments are done at a very small, 5 ml scale, but this is still a substantial amount of material. Catalysts, particularly palladium catalysts, are very expensive. The material is needed for nuclear magnetic resonance (NMR) or mass spectroscopy to analyze the chemical properties.

With capillary electrophoresis and its microvials and microtiter plate, 96 reactions with 96 different conditions can be performed and then analyzed at the same time. The advantages are less material—only 100 µl of solution instead of 5 ml—saving reagent costs and time.

Two isomers with completely different chemical properties are produced in this reaction (Figure 43); these properties must be identified—how much of this form and how much of that form, similar to chiral—and separated from the starting material. The separations can be done in HPLC with reverse phase chromatography, but for capillary electrophoresis, the molecules are neutral and the normal ionic effect cannot be used.

The nitrogen in the isomers can be protonated by adding trifluoro acid, a relatively strong organic acid, that will create ions. Each nitrogen has a slightly different affinity, so the charge will be slightly different, the ions will move with the electric field, and they can then be separated by capillary electrophoresis.

Dimethylformamide (DMF), an organic solvent, was used in this reaction, and so that the reaction is not disturbed, it must be separated in organic solvent to preserve the organic environment. The capillary tube is simply filled with DMF. Using organic solvents in place of water is called nonaqueous capillary electrophoresis.

An advantage of nonaqueous electrophoresis using organic solvents is there will be no charge on the surface of the fused silica tubes. Because the silanol groups are also present in ionization, there is no electroosmotic flow, only electrophoresis, and everything is reproducible.

Every chromatogram has four peaks to analyze. Each time the CE instrument takes only 10 to 20 nl of solution from the 100 ml tube—a negligible amount of material.

Adjustments can be made during the reaction; if the reaction is not progressing well, the temperature can be adjusted during the run and so forth.

Figure 44 shows 96 different conditions that were tried with eight different catalysts, including a controlled experiment with no catalyst, and 11 different solvent conditions. A peak area from each isomer, A and B, was integrated and plotted against the 96 sample tubes to immediately show the two best conditions for synthesis (about 90%).

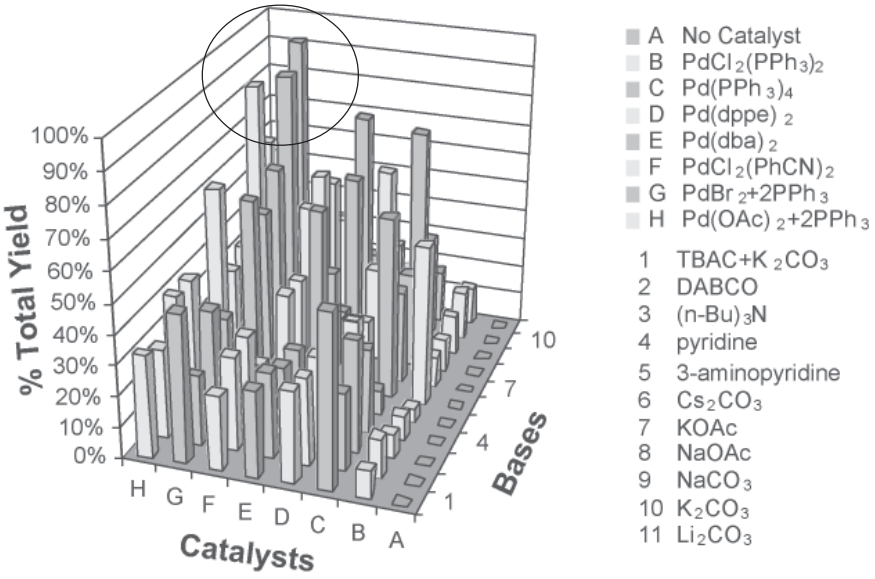


Figure 44. Combinatorial synthesis (one experiment instead of 96).

The light bar in the circle is the optimum condition that was found after three months of trial and error with a limited number of experiments by an organic chemist. Superior results were found by capillary electrophoresis because of all the conditions that can be tried in one run.

Using the ratio of the peaks, it can be seen that some conditions are favorable for producing isomer A and some conditions are favorable for producing isomer B (Figure 45).

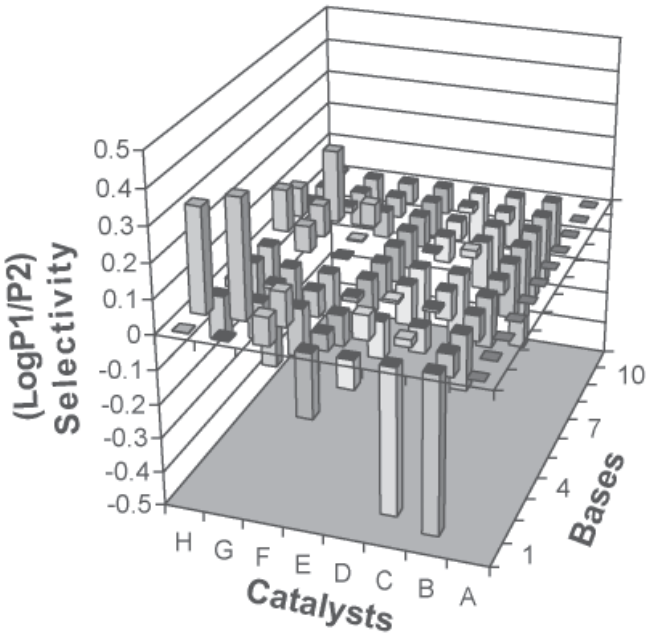


Figure 45. Combinatorial analysis (produces total yield, stereospecificity, kinetics).

A combination of the two plots shows the best synthetic root for the particular product.

Many samples can be analyzed at one time, and because the reaction volume is reduced, it becomes practical to run many volumes, reducing development costs.

NOTES

Capillary Electrophoresis

Self-Evaluation

1. Basic Mechanism and Operation

- What is electrophoresis?

- What are the advantages and disadvantages of using capillary electrophoresis?

- List factors that influence separation and indicate which factor is the easiest to control.

- What is electroosmotic flow and how is it a factor in capillary electrophoresis?

- What is electroosmotic velocity?

- How do you reduce electroosmotic flow?

- Define apparent mobility.

- Restate apparent mobility using the rower, boat, and stream analogy, explaining what each part represents.

- Viscosity of water changes by about _____% every _____° C, resulting in varying speeds that the molecules arrive at the detector. How can viscosity be controlled if the temperature in the laboratory can fluctuate 5° C in one day?

_____

_____

_____

_____

- A micelle is a fancy term for a _____.

- MEKC is a modification of CE used to separate _____ compounds.

- How do micelles separate compounds in micellar electrokinetic chromatography?

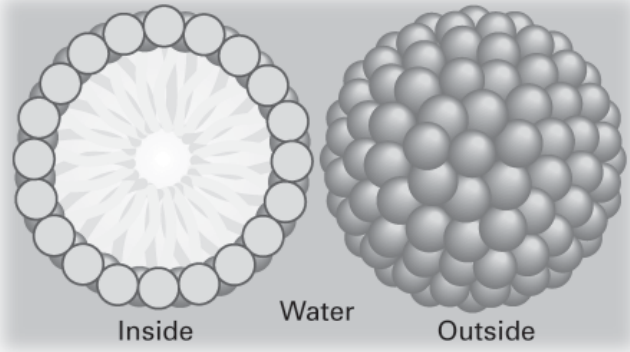
_____

_____

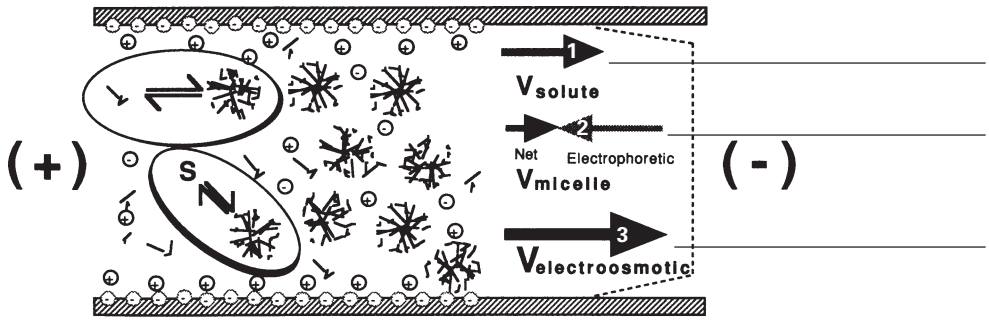
_____

_____

- Label the monomers and polar, nonpolar, and hydrophobic areas on the micelle below.



▪ Label arrows 1–3 of MEKC diagram.



▪ What are the advantages of using MEKC?

▪ Which variables/elements can be changed to get better separation with MEKC?

2. Methods for DNA Sequencing and Genetic Analysis

▪ Define DNA.

▪ Why can't DNA molecules be separated with CZE?

▪ The snakelike (or reptilelike) movement of DNA molecules through the polymer network is called

_____.

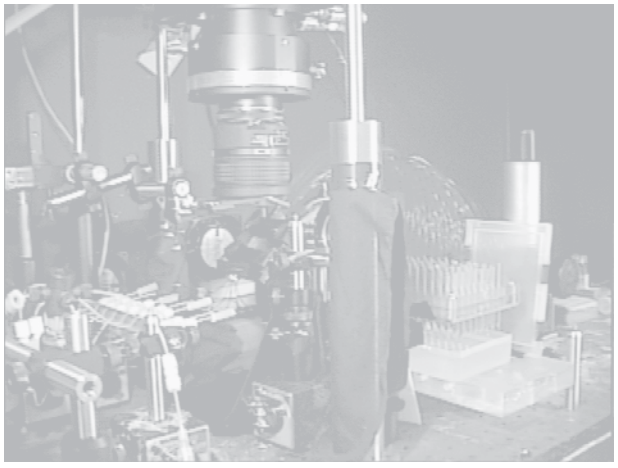
▪ What is a problem with using frigid crosslinked gels of the past?

▪ What are the benefits of the newer entangled polymers?

▪ Why would you use multiplexed capillary electrophoresis?

▪ What is the new separation matrix for capillary gel electrophoresis?

▪ Label the elements of a DNA sequencing instrument.



DNA sequencing instrument.

▪ _____ was the driving force for changes in CE instrumentation and processes.

▪ How is capillary gel electrophoresis used for genetic analysis?

▪ What is the standard acceptable number of genetic regions needed for DNA fingerprinting?

▪ How are special entangled polymers used to separate DNA?

▪ What is the target resolution for DNA?

▪ What are optimal separation results and how would you achieve them?

▪ What happens if the column is not coated?

▪ What are the benefits of using polymer PEO over PA?

▪ What are the symptoms and consequences of improper sample handling?

▪ How do we know that a peak is DNA and not some other type of contaminant?

- What are different ways of genotyping and the advantages and disadvantages of each?

- Why is it almost impossible to deliberately spoil a DNA sample to give misidentification?

- What is spiking, how is it different from other DNA fingerprinting techniques, and what are its advantages?

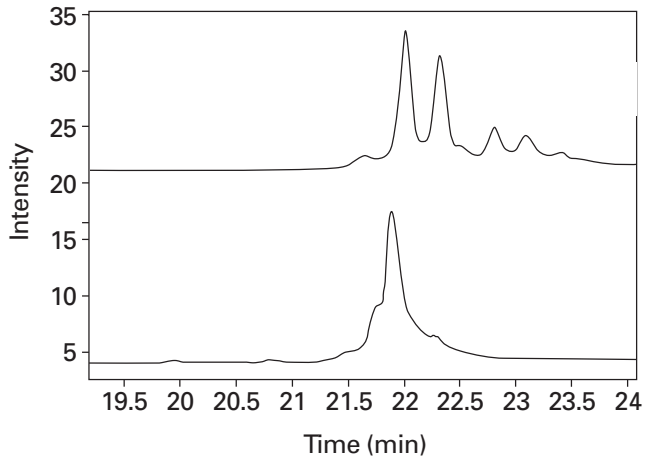
- What is the importance of temperature-dependent polymers and how do they work?

▪ What is the single point mutation process (SNP)?

▪ Define homoduplex.

▪ Define heteroduplex.

▪ Label the homoduplex and heteroduplex results on this electropherogram.



- Why is it important to have a temperature gradient in SNP?

- What are the advantages of using SNP over other types of genotyping?

- How many separate suspect samples can be DNA fingerprinted in one 96-capillary electrophoresis run?

3. Combinatorial Separations

- What areas need high throughput for chemical separation?

- What are the performance challenges the 96-well capillary absorption detector can have and how can these problems be solved?

- What is the concentration process?

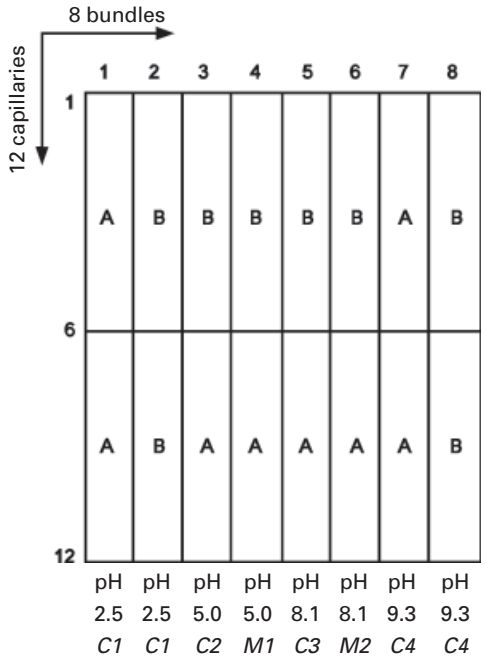
- What is a protein?

- Define peptide and explain how a peptide fragment is made.

▪ What is a reliable way to identify proteins?

▪ How are acids and bases separated?

▪ Using the example below, describe how peptide mapping takes advantage of the combinatorial separation conditions enabled through the use of 96-well CE.



▪ How can HPLC and multiplex CE complement each other?

▪ What are the beneficial results gained by using HPLC and multiplex CE in tandem?

▪ What is the process of using complementary HPLC and multiplex CE?

▪ What are chiral molecules and why is it important to separate them?

▪ Describe both an expensive and an inexpensive way to separate chirals.

▪ How does an additive like cyclodextrin separate chirals?

▪ Explain the optimization of the chiral separation solution through combinatorial CE.

▪ What is the goal of synthesis?

- What are the benefits of the combinatorial synthesis process using high speed, high throughput?

- What is nonaqueous electrophoresis and what are its advantages?

- How has using combinatorial high speed, high throughput nonaqueous electrophoresis changed the synthesis process?